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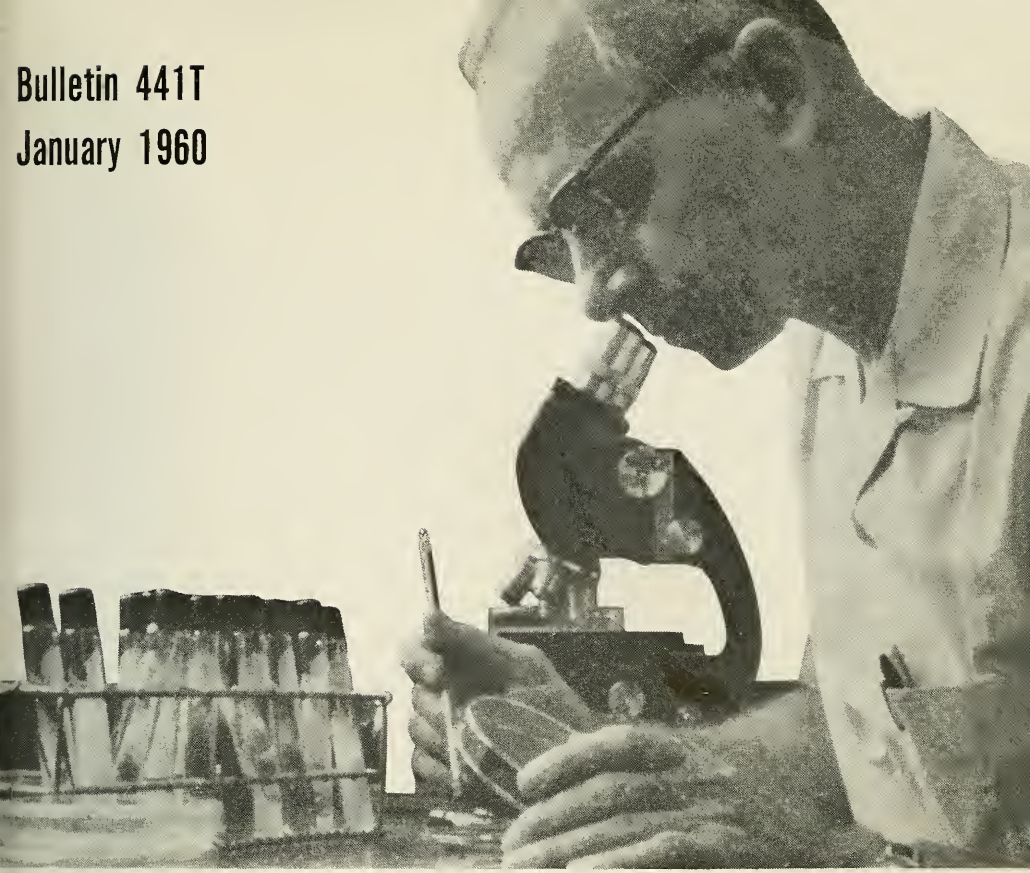

The Production of Carotene by *Phycomycees blakesleeanus*

WEST VIRGINIA UNIVERSITY AGRICULTURAL EXPERIMENT STATION

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by
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The Production of Carotene by *Phycomyces blakesleeanus*

VIRGIL GREENE LILLY, H. L. BARNETT and R. F. KRAUSE

Introduction

THE carotenoids comprise a large group of related polyene compounds, most of which contain 40 carbon atoms per molecule. The more unsaturated carotenoids are yellow or red while a few highly reduced members are colorless. These compounds may be separated into two subgroups: (1) hydrocarbons (carotenes), and (2) their oxygenated derivatives (carotenols, etc.). In addition to green plants, some species of bacteria and fungi synthesize carotenoids.

The general designation polyenes is used to refer to the compounds having 40 carbons whether colored or not. The term carotenoid is used for the colored members of the polyenes. Carotene is used to designate the mixture of pigments found in various organisms when the chief carotenoid is β-carotene. Specific carotenoids are referred to by their specific trivial names, e.g., γ-carotene.

The fungi offer a number of advantages for the study of cartenogenesis. Many species which produce carotenoids grow rapidly and may be cultured in the laboratory under controlled nutritional and environmental conditions. The synthesis of carotenoids by fungi is being intensively studied from the following viewpoints: (1) The effects of nutritional and environmental conditions upon carotenogenesis; (2) The role of the carotenoids in the fungi which produce them; (3) The pathways of biosynthesis; (4) The production of β-carotene-C^{14} for chemical and nutritional studies; and (5) The microbiological production of β-carotene for use as provitamin A for animals.

General Literature Review

Goodwin (1952, 1954, 1955 and 1958) has summarized much of the work on carotenoids. The literature pertinent to viewpoints 1 and 4 (above) will be reviewed in connection with the experimental work presented in this Bulletin. The others will be reviewed briefly here.

The role of carotenoid pigments in fungi is unknown. In many species, the carotenoids accumulate in the fruiting structures. This association of carotenoids and sporulation led Robinson (1926), who worked with *Pyronema confluens,* to conclude that there was a direct relationship between carotenogenesis and reproduction. It is difficult to separate the processes of carotenogenesis and sporulation in many fungi because the same stimulus, light, is required to initiate both processes. Carlile and Friend (1956) used a mutant of *P. confluens* which produced no detectable concentration of carotenoids and which sporulated when exposed to light. They concluded that light stimulated two distinct processes in *P. confluens,* carotenogenesis and sporulation, and that the two processes were not related.

Mixed + and − clutures of *Choanephora cucurbitarum* produce from 10 to 15 times as much carotene as either sex grown separately (Barnett *et al.* 1956). Apparently each sex produces a substance(s) which stimulates carotenogenesis in the other. Enhanced production of carotene occurred in both sexes separated by a cellophane membrane (Barnett and Lilly 1956). Hesseltine and Anderson (1957) have extended these observations to other species of the Choanephoraceae. Increased synthesis of carotene occurred when + and − isolates of *different* species were cultured together. Incomplete hybridization occurred between certain + and − isolates of various heterothallic mucors (Blakeslee 1915). It is not known whether the substances which stimulate carotenogenesis and sexual reproduction are the same.

The study of the pathways involved in the biosynthesis of the carotenoids is a difficult one. Organisms usually synthesize more than one carotenoid, and the carotenoids synthesized may be a function of the age of the organism and the nutritional and environmental conditions during synthesis (Grob 1953; MacKinney *et al.* 1953). The possibilities of single, multiple, and alternative pathways must be considered as well as possible interconversions among the carotenoids in the cells. Various schemes for carotenogenesis have been proposed.

Porter and Lincoln (1950) proposed that a hydrocarbon resulting from the condensation of 8 isoprene units was the parent compound from which the various carotenoids were formed by the processes of dehydrogenation, oxidation, cyclization and isomerization. Genevois (1954) modified this hypothesis by proposing that isoprenol, as well as isoprene, is involved in the primary condensation; this modification allows the introduction of hydroxyl groups early in the scheme. Two assumptions are inherrent in the above scheme: (1) that interconversion of carotenoids occurs, and (2) that a C_5 (or C_6) compound(s) is the precursor of the

carotenoids. Most recent investigators are of the opinion that the various carotenoids are synthesized by parallel pathways rather than being derived from a single parent hydrocarbon.

A number of C_5 and C_6 compounds have been shown to stimulate carotenogenesis in fungi. Reichel and Wallis (1956) found 3-hydroxyvaleraldehyde (C_5), 3-methylcrotonic acid (C_5), 3,3-hydroxymethylglutaric acid (C_6), and 3-hydroxyisovaleric acid (C_5) to increase the production of β-carotene by *Phycomyces blakesleeanus*. No evidence was given which indicated that any of these compounds was incorporated intact into the β-carotene molecule.

Among the simple compounds which have been shown to be precursors of β-carotene, acetate appears to be the most important. Schopfer and Grob (1952) showed that *P. blakesleeanus* synthesized no more than a trace of β-carotene when cultured on a lactate medium. The addition of acetate to the lactate medium allowed the synthesis of β-carotene. Grob *et al.* (1951) showed that 1-C and 2-C of C^{14}-labeled acetate were incorporated equally into the molecule of β-carotene. *Mucor hiemalis* was shown to incorporate both carbon atoms of acetate equally into β-carotene (Grob and Bütler 1954).

The importance of acetate in the biosynthesis of β-carotene is noted above. The question of a suitable C_5 or C_6 precursor for the condensation steps leading to a C_{40} compound remains unsettled. Recently, Skeggs *et al.* (1956) found an acetate replacing factor for certain *lactobacilli* in distillers' solubles and other complex natural substances. The chemical nature of this compound was elucidated by Wolf *et al.* (1956, 1957) and confirmed by synthesis. This compound was shown to be β-hydroxy-β-methyl-δ-valerolactone (trivial name, mevalonic acid). Tavormina *et al.* (1956) found 43 per cent of the radioactivity of dl-mevalonic acid-2-C^{14} to be incorporated into cholestrol synthesized by rat liver homogenates. This indicates almost complete incorporation of the intact molecule into cholestrol if it is assumed that only one of the isomers is active. It is possible that the long-sought active isoprene precursor of the carotenoids has been discovered. Grob (1957) reported some experimental results with *M. hiemalis* which indicate that this acetate replacing factor is incorporated into β-carotene. Highly radioactive β-carotene was obtained from malonic acid inhibited cultures to which *dl*-2-C^{14} mevalonic acid was added. Unfortunately, only a few experimental results were reported. Braithwaite and Goodwin (1957) have reported on the incorporation of mevalonic acid into β-carotene synthesized by *P. blakesleeanus*. Much additional work remains to be done on the pathways of biosynthesis of the carotenoids.

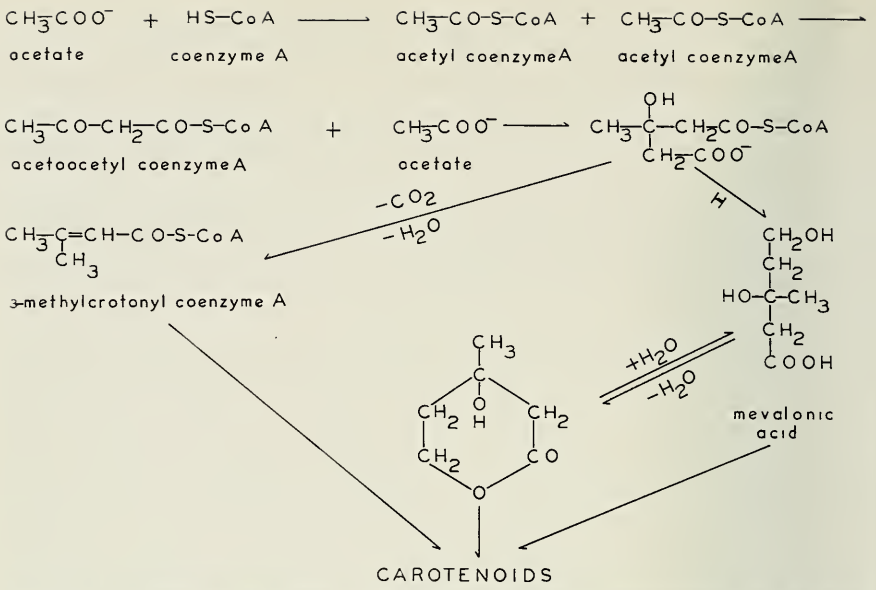

FIGURE 1. Possible pathways for the biosynthesis of carotenoids from acetate.

If mevalonic acid is the direct precursor of carotenoids which arise by the condensation of 8 molecules (with the elimination of a terminal carbon), it would be expected that 5 carbon atoms in this active isoprene moiety would enter the carotenoid molecule equally. More work with this precursor labeled in various positions will be necessary to substantiate this hypothesis.

Grob (1956, 1958) and Grob and Bütler (1955, 1956) have proposed a number of schemes for the biosynthesis of β-carotene. A modification of these hypotheses is given in Figure 1.

The discovery of Barnett *et al.* (1956) that the amount of carotenoids (mostly β-carotene) synthesized by *Choanephora cucurbitarum* was increased from ten- to fifteenfold by culturing the two sexes of this fungus together reawakened interest in this problem. The application of this discovery was extended by Hesseltine and Anderson (1957). Some of the results obtained in shake culture studies have appeared (Anderson *et al.* 1958; Ciegler *et al.* 1958, 1958a). Carotene yields of 8 to 9 mg./g. dry mycelium of mixed cultures of + and − sexes of *Blakeslea trispora* have been reported. With one exception (Booth 1957), these values are many times greater than any reported for green plants (Goodwin 1954).

The work reported in this Bulletin is a portion of a larger study on the production and role of carotenoids in fungi. The purpose of this

work was twofold: (1) To develop a suitable medium and to define the environmental conditions for the production of good yields of β-carotene by an isolate of *P. blakesleeanus*; and (2) to use this information as the basis of a method for the biosynthesis of β-carotene-C^{14}. Both phases of the study were made concurrently. *P. blakesleeanus* was selected on the basis of available information and our familiarity with this species.

Materials and Methods

This series of investigations was started in November, 1954. Various modifications were introduced into the experimental procedures as various nutritional and environmental factors were studied. For this reason, it has been necessary to include a certain amount of detail in the description of the various groups of experiments. The order of presentation does not always follow the order in which the experiments were carried out.

Four isolates of *Phycomyces blakesleeanus* Burgeff were tested. The laboratory numbers and sexual type are given below:

No.	*Sexual Type*
103	+ (strong)
104	− (strong)
1036	− (weak)
1177	+ (weak)

Isolates 103 and 104 have been maintained in the culture collection for more than 20 years. Presumably they came from the Centraalbureau voor Schimmelcultures, Baarn, but the record has been lost. Two cultures were isolated locally, 1036 from the grapefruit rind and 1177 from dead leaves. Unless otherwise specified, all of the experimental work was done with isolate 104.

Spore suspensions were used as inoculum. Cultures used for the production of spores were grown at 25° C. for 2 to 3 weeks on 50-ml. of agar medium in 1-liter Erlenmeyer flasks. The sporulation medium contained per liter 20 g. of malt extract (Difco), 2 g. of yeast extract (Difco), and 20 g. of agar. The pH was adjusted to 6. The spores were collected in 50 ml. of sterile distilled water, dispensed into screw cap test tubes, and stored in the freezing compartment of a refrigerator until used. The spore suspensions were tested for contaminating organisms before being stored. Spores of *P. blakesleeanus* stored in this way are viable for several years, however, spores less than 6 months old were used in the experiments.

The basal synthetic medium which evolved as a result of this study had the following composition[1]: D-glucose, 10 g.; potassium acetate, 0.72 g.; $(NH_4)_2SO_4$, 1.0 g.; KH_2PO_4, 1.0 g.; $MgSO_4 \cdot 7H_20$, 0.5 g.; Fe (III) and Zn (II), 0.1 mg. each; Mn (II) 0.05 mg.; Ca (II), 10 mg.; thiamine chloride, 100 μg.; and double distilled water *q.s.* one liter. The basal medium was machine pipetted in 25-ml. aliquots into acid-washed 250-ml. Pyrex Erlenmeyer flasks. The flasks were capped with 50-ml. beakers. The pH of the medium was adjusted to 5.75 before autoclaving 15 minutes at 121° C. The pH value of each lot of medium was redetermined after autoclaving (initial pH). The flasks were inoculated with one drop of a spore suspension prepared as described above. Many variations in the composition of the medium were tried. These are noted in connection with some of the individual experiments.

In most of the early work the cultures were incubated in constant temperature rooms at 25 ± 1° C. unless otherwise noted. At first the amount of light received by the cultures was not controlled closely, but when the importance of light was recognized the cultures were illuminated 24 hours daily with white fluorescent lamps, at a light intensity of 50 to 60 foot-candles. In experiments where C^{14}-compounds were used, the cultures were incubated in stainless steel hoods in the isotope laboratory of the West Virginia University Physics Department. A temperature of 25 ± 1° C. and 24-hour illumination at 50 to 60 foot-candles were used in these experiments.

A sufficient number of flasks, usually from 20 to 40, were used in each experiment so that daily determinations of the carotene content of the mycelium could be made. In addition, the dry weight of the mycelium, the pH of the culture filtrate, and the amount of residual glucose in the filtrate were determined daily. In experiments with C^{14}-compounds the residual radioactivity of the medium was determined. In a few experiments the residual nitrogen in the medium and the lipid content of the mycelium were determined. The mycelium from duplicate flasks was collected individually on a fine mesh cloth, washed with distilled water, placed in aluminum weighing cups, and dried for 24 hours at 100° C., and weighed on an analytical balance; the dry weights reported are averages of two determinations. The pH values of the culture filtrates were determined with a laboratory model Cambridge pH meter.

[1]The D-glucose used was anhydrous Cerelose (Corn Products Refining Corp.). The other chemicals used were C.P. or A.C.S. grade. The composition of the medium given above refers only to the major constituents and does not include the impurities present in the chemicals or distilled water used. Twice this concentration (2 ×) of micro elements was used in much of this work. See section on micro elements, p. 50 for a discussion on the effect of micro element concentration on carotenogenesis.

The residual glucose was determined by the Folin-Wu method (Keller 1951). Residual nitrogen in the medium was measured by a micro Kjeldahl procedure. The lipid content was calculated on the basis of the difference in weight of dry mycelium before and after exhaustive extraction with diethyl ether in a Soxhlet apparatus.

Carotene was determined by measuring the optical density of diethyl ether extracts of mycelium with a Beckman model DU spectrophotometer which had been calibrated with a sample of pure β-carotene. All of the results for carotene concentration are reported as β-carotene. The mycelium was ground with peroxide-free diethyl ether in a mortar to extract the carotenoids according to the procedure of Garton *et al.* (1951). The grinding was continued until the diethyl ether extract was colorless. The pooled extracts were kept in glass stoppered graduates made of Pyrex low-actinic glass until the optical density was measured. In the early experiments, the carotene content of 4 replicate cultures was determined individually. The variation in yield among cultures was small (± 5%). In the later experiments, the carotene content was determined for pooled duplicate cultures. The carotene content is reported as μg./culture, or as μg./g. dry mycelium.

In many experiments, additional acetate was added during incubation. The details will be found in connection with the experiments on acetate utilization. When radioactive acetate (or other radioactive compounds) was used, the radioactivity of the medium was determined from time to time. Acetate-1-C^{14} or acetate-2-C^{14} was added to the cultures in amounts ranging from 1 to 40 μc./culture. At the same time this amount of radioactive acetate was added to 2 uninoculated control flasks. Small samples (0.5 ml.) of medium were removed with sterile pipettes and placed in screw capped test tubes. Samples not analyzed immediately were stored at −20° C. until counted. Aliquots (0.1 ml.) of the media were pipetted onto planchettes and neutralized (phenophthalein) with sodium hydroxide. The samples were then dried under infrared lamps and counted with a Tracerlab windowless gas flow counter. The radioactivities are reported as direct counts per minute (C.P.M.) corrected for background. The radioactivity of β-carotene-C^{14} was determined with the same instrument.

Experimental Results

The experimental work is presented in two sections. Part I includes the experiments on the effect of nutrition and environment on the production of carotene. Part II describes the production and isolation of pure β-carotene-C^{14}. The ultimate goal was to produce β-carotene-C^{14} with sufficiently high specific activity for animal experimentation.

PART 1: The Effects of Nutrition and Environment on Carotenogenesis

The experiments reported in this part of the Bulletin were primarily concerned with the development of a suitable basal medium and the evaluation of environmental factors for the production of high yields of carotene. In most experiments, the relations among growth (dry weight of mycelium) pH of the culture medium, glucose utilization, and the yield of carotene were determined as functions of the time of incubation.

ACETATE MEDIA

Phycomyces blakesleeanus (Schopfer and Grob, 1952) is able to grow on a medium containing acetate as the sole source of carbon. Friend *et al.* (1955) reported growth also on an acetate medium and that β-ionone stimulated carotenogenesis on this medium.

In some initial experiments in this laboratory, the glucose in the basal medium was replaced by 9.8 g./l. of potassium acetate. *P. blakesleeanus* in still culture grew poorly on this medium, and the yield of carotene was low compared with that obtained later on other media. The dry weight of mycelium (22 days) ranged from 23.5 to 15.4 mg./culture when the initial pH values of the media were 4.96 and 6.18. The corresponding yields of carotene were 225 and 330 μg./g.

Other experiments, designed to determine whether aeration and agitation would improve the yields of mycelium and carotene when the acetate-ammonium sulfate medium was used, involved the use of 9-liter serum bottles as culture vessels. When sterile air was introduced at the bottom of the bottles through a glass tube the yields of mycelium and carotene were still low. Somewhat better results were obtained when the culture medium was neutralized daily (pH 6.8). Phenol red was used as an internal indicator.

Phycomyces blakesleeanus grew on a medium containing acetate as the sole source of carbon. However, growth was limited and the carotene content of the mycelium was low.

AERATED CULTURE

Goodwin (1953) states that *P. blakesleeanus* cannot be grown in shake culture. However, Rauch and Peiker (1950) had reported more rapid growth in an agitated glucose-ammonium sulfate medium than in still culture.

We encountered no difficulty in culturing *P. blakesleeanus* in 6-liter lots of the basal medium under the conditions used with the acetate

medium. The yield of mycelium was lower per unit volume of medium than that obtained in 250-ml. flasks. The yield of carotene was disappointingly low. Further work with this procedure was abandoned. It is not known why agitation reduced the yield of carotene. Agitation also reduced the yield of carotene by *Penicillium sclerotiorum* (Mase *et al.* 1957). *Blakeslea trispora,* on the other hand, produced high yields of carotene in shake culture (Anderson *et al.* 1957).

Phycomyces blakesleeanus is capable of growing in aerated culture; little carotene is produced under these conditions.

GLUCOSE-ACETATE MEDIA

It is well known that some carbon sources are not utilized, or are utilized poorly, when they are supplied as the sole source of carbon but are utilized in the presence of a second source of carbon (Lilly and Barnett 1953; Waters *et al.* 1953; Johnson and Johan 1954; Blair and Pigman 1954; and Machlis 1957). Leonian and Lilly (1940) and Friend *et al.* (1955) found acetate, and the salts of various other organic acids, to increase the amount of growth of *P. blakesleeanus* when cultured on a basal medium containing glucose and an ammonium salt. Friend *et al.* (1955) found carotenogenesis to increase when the concentration of added acetate was less than 0.02 *M,* and to decrease at higher concentrations of acetate.

More detailed observations were made on the effect of acetate concentration in the series of experiments reported below. The various concentrations of potassium acetate added to the basal glucose-ammonium medium were: 0.0, 0.0025, 0.005, 0.01, 0.02, and 0.04 *M.* (0.0, 0.245, 0.49, .98, 1.96, and 3.92 g./l.) The production of mycelium and carotene was studied as a function of the time of incubation. The pH values of the culture medium and the quantity of glucose remaining in the medium were determined for each harvest. The essential data are given in Figures 2 to 5. Two $\times$ concentrations of micro elements were used in all of the experiments reported in this section.

The effects of adding various quantities of potassium acetate to the basal glucose-ammonium sulfate medium on the dry weight of mycelium produced by *P. blakesleeanus* are illustrated in Figure 2. Growth was poor in the control medium (no added acetate). The addition of acetate, 0.0025, 0.005, 0.01, and 0.02 *M,* resulted in a regular increase in the maximum dry weight of mycelium produced per flask. The early rate of growth was increased as the concentrations of acetate were increased to 0.02 *M.* When the concentration of acetate was increased to 0.04 *M* the rate of growth and the weight of mycelium produced were

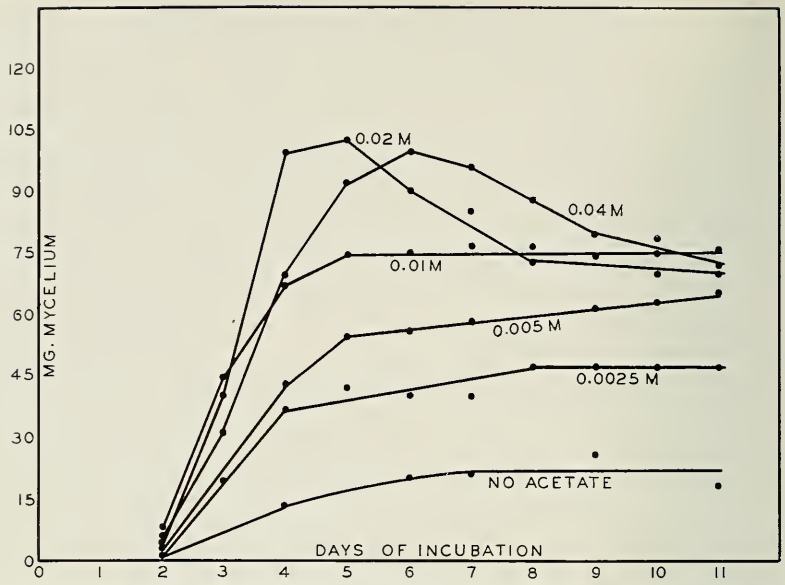

FIGURE 2. The effects of adding various concentrations of potassium acetate to a glucose-ammonium sulfate medium on the dry weight of mycelium produced per culture.

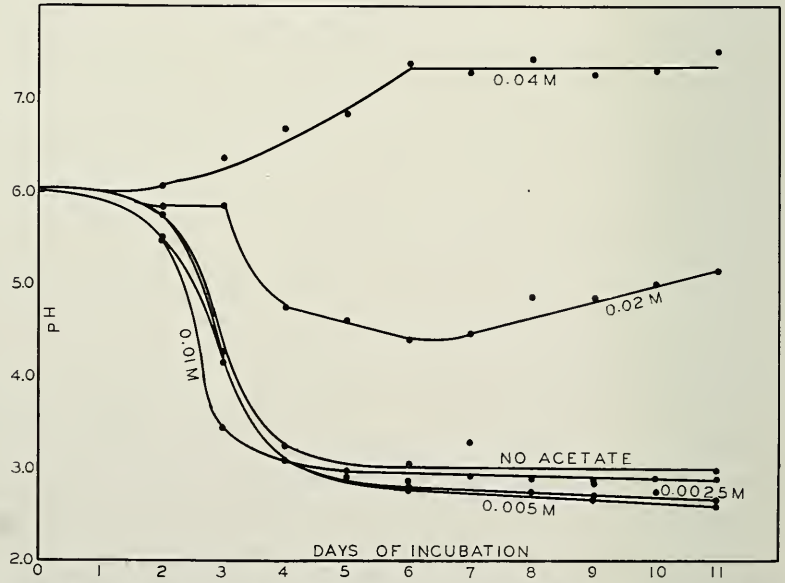

FIGURE 3. The effects of adding various concentrations of potassium acetate to a glucose-ammonium sulfate medium on the changes in pH values of the culture media during incubation.

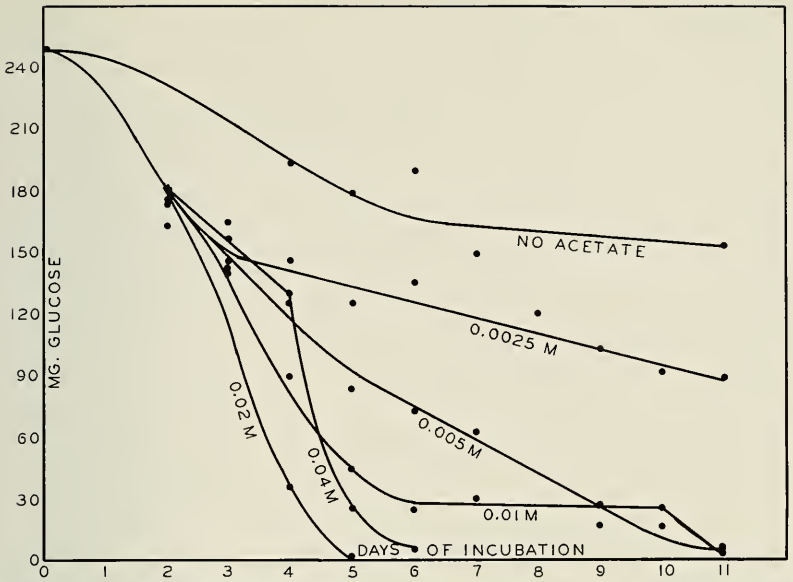

FIGURE 4. The effects of adding various concentrations of potassium acetate to a glucose-ammonium sulfate medium on the rate and amount of glucose utilized during incubation.

slightly less than when half this quantity of acetate was used. Sporangiophores from 2 to 3 inches long were produced by the end of 5 days of incubation when the concentrations of acetate were 0.02 and 0.04 *M*.

The effects of various concentrations of acetate on the pH values of the culture media are given in Figure 3. It is noteworthy that the pH values of media containing 0.0025, 0.005, and 0.01 *M* were lower than those of the control medium to which acetate was not added. The increased production of mycelium (Figure 2) in media containing these concentrations of acetate cannot be attributed to the buffer action of potassium acetate. The use of higher concentrations of acetate (0.02 and 0.04 *M*) did result in higher pH values of the culture media.

The concentration of acetate added to the basal medium affected the rate of glucose utilization. Less than half of the glucose in the control medium was utilized in 11 days (Figure 4). Glucose was present in the control medium after 34 days of incubation. As the concentrations of acetate were increased from 0.0025 to 0.02 *M*, the rate at which glucose was utilized increased. However, glucose utilization was slower in the presence of 0.04 than 0.02 *M* acetate.

The amounts of carotene accumulated per culture increased as the concentrations of acetate were increased from 0.0025 to 0.01 *M* (Figure 5). Acetate concentrations of 0.2 and 0.04 *M* resulted in the accumu-

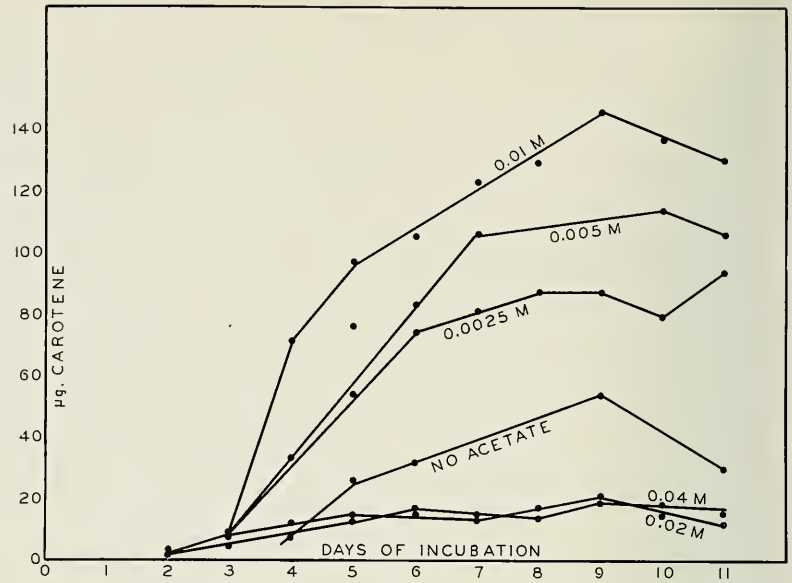

FIGURE 5. The effects of adding various concentrations of potassium acetate to a glucose-ammonium sulfate medium on the accumulation of carotene.

lation of less carotene per culture than in the control cultures (no acetate).

It appears that the control cultures and those receiving from 0.0025 to 0.01 *M* acetate accumulated about the same amount of carotene per gram of dry mycelium. Thus, the concentrations of carotene after 9 days of incubation were: Control, 2,080; 0.0025 *M*, 1,840; 0.005 *M*, 1,800; and 0.01 *M*, 1,960 μg./g.

In view of these results, it is apparent that the external factors which increase carotenogenesis in *P. blakesleeanus* are common to the control cultures as well as those grown in the presence of 0.01 *M* or less acetate. These favorable external conditions include pH values of the culture medium less than 3 (by the fourth day of incubation), and the presence of residual glucose in the medium. These conditions also are characterized by a suboptimal production of mycelium and the absence of appreciable asexual sporulation. The addition of acetate in concentrations greater than 0.01 *M* results in the production of more mycelium, higher pH values, and normal asexual sporulation. Only a little carotene (ca 200 μg./g.) was synthesized, or accumulated, under the conditions which resulted in "normal" growth and asexual sporulation.

More detailed experiments were made with media in which the initial concentrations of acetate were 0.006, 0.007, 0.009, 0.01, 0.012, 0.014, and 0.016 *M*. A portion of the data for weights of mycelium produced

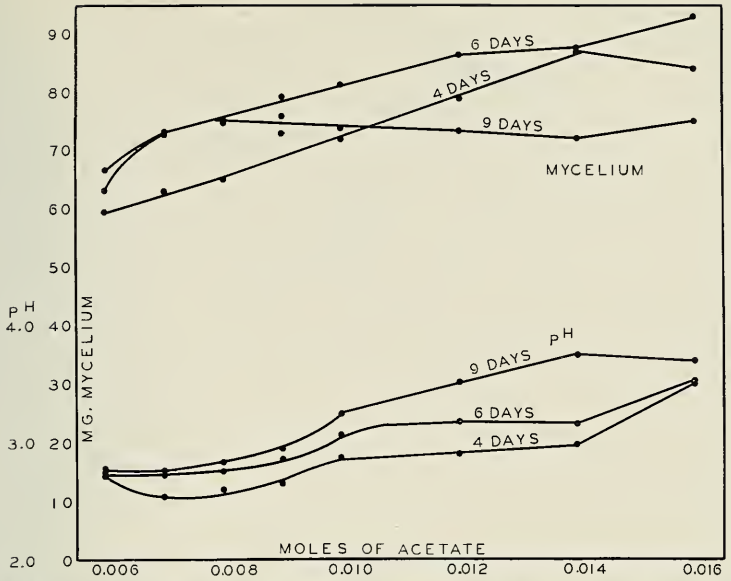

FIGURE 6. The effects of potassium acetate concentration on the production of mycelium and the change in pH values of the culture medium after 4, 6, and 9 days of incubation.

and pH values of the nutrient solutions are given in Figure 6. By the fifth day of incubation sporangiophores began to develop in the cultures growing in media containing 0.01 to 0.016 *M* acetate. Their numbers and length increased as the concentration of acetate increased. However, sporangiophore development in the medium containing 0.016 *M* acetate was not as luxuriant as in the medium containing 0.02 *M* acetate.

Some of the data for carotene production on a flask basis are given in Figure 7. The best yields were obtained when the acetate concentration was between 0.006 and 0.009 *M*. On the basis of carotene production per unit weight of mycelium (μg./g.), 0.006 *M* acetate was the most efficient (Figure 8). On the basis of these experiments, and others which are not reported, an initial concentration of 0.0075 *M* acetate was selected for the basal medium. The results from a typical experiment in which the basal medium was used are given in Figure 9. The environmental conditions used in this experiment were continuous light (50 to 60 foot-candles) and a temperature of 25° C. It will be noted that more than half (47.3 mg.) of the final dry weight of mycelium (67.5 mg.) was produced by the end of the third day of incubation while 21 μg. of carotene/culture was present at this time, or about 12 to 13 per cent of the quantity present in the mycelium after 7 to 10 days of incubation. The pH value of the nutrient solution had fallen from 5.8 (initial pH)

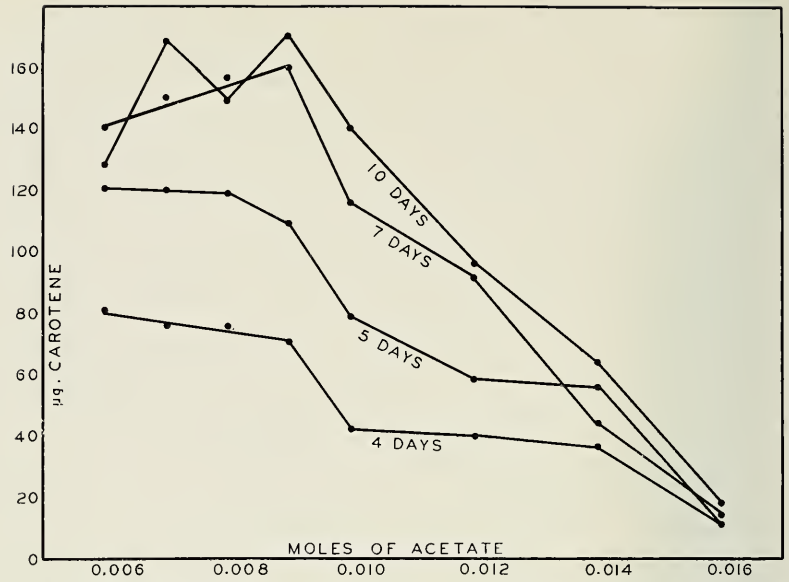

FIGURE 7. The effects of potassium acetate concentration on the accumulation of carotene (µg./culture) after 4, 5, 7, and 10 days of incubation.

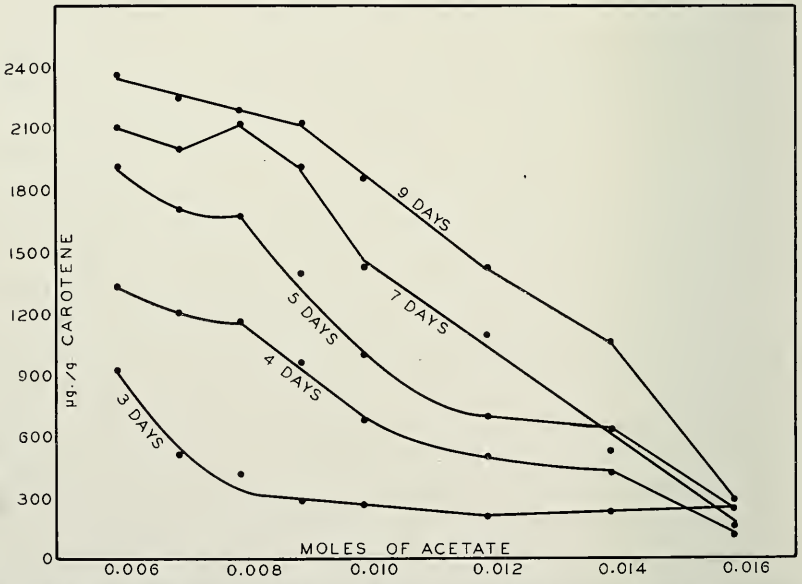

FIGURE 8. The effects of potassium acetate concentration on the accumulation of carotene (µg./g.) after 3, 4, 5, 7, and 9 days of incubation.

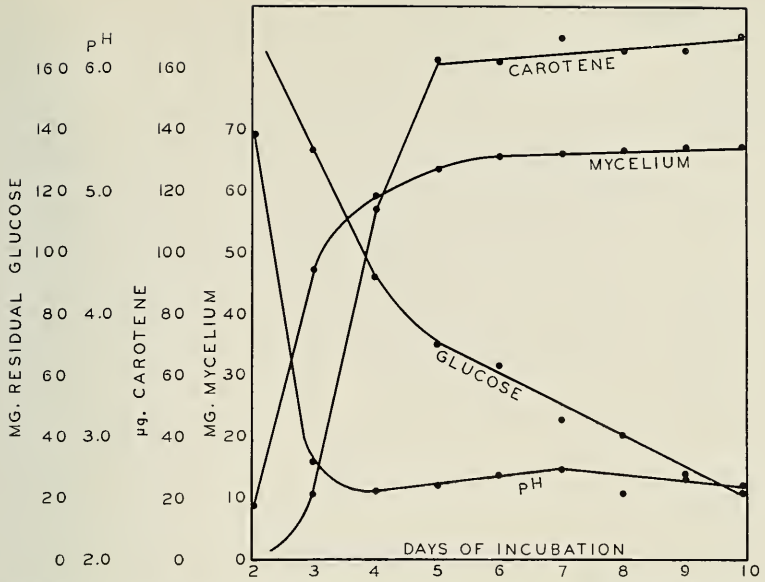

FIGURE 9. Growth, pH of the culture medium, residual glucose, and amount of carotene accumulated between the second and tenth day of incubation.

to 2.8 after 72 hours. Some glucose was present in the medium after 10 days of incubation.

It became of interest to determine whether the acetate in the basal medium was utilized by *P. blakesleeanus*. The amount of residual acetate in the medium after different times of incubation was determined by a microdiffusion method using Conway vessels. In later experiments, the decrease in radioactivity of culture filtrates was measured to determine the amount of acetate utilized. One μc. of acetate-1-C^{14} per flask was added to the basal medium before inoculation. This quantity of labeled acetate was added to control flasks which were not inoculated; the radioactivity of the control flasks did not change with time. The radioactivity of the culture filtrates was determined daily (Figure 10). After 3 days of incubation, the culture medium contained about 18.5 per cent of the radioactivity added. By the fourth day, only 5 per cent of the original radioactivity remained in the culture medium. If it is assumed that the carrier and labeled acetate were utilized at the same rate (absence of an isotope effect), any direct effect of the 0.0075 *M* acetate in the basal medium must occur within the first 4 days of incubation. Control experiments in which labeled acetate was added to uninoculated media (pH 2.5) showed that little or no radioactivity was lost within a week. It was concluded that the decrease in radioactivity of the medium which

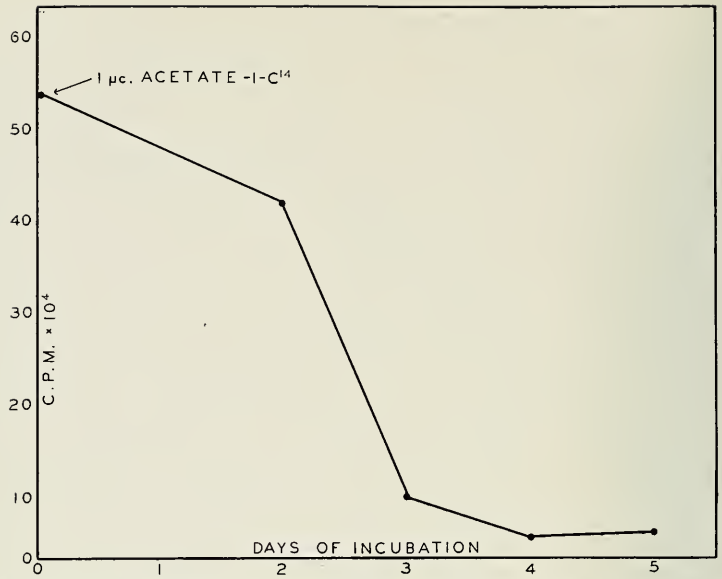

FIGURE 10. The decrease in radioactivity with the time of incubation of a culture medium to which acetate-1-C^{14} had been added at the time of incubation.

occurred when labeled acetate was added to cultures of *P. blakesleeanus* was due to utilization and not to loss of acetic acid.

The addition of acetate to the glucose-ammonium sulfate medium modified its properties for P. blakesleeanus. Concentrations of acetate up to 0.02 *M increased growth and utilization of glucose. The carotene yield per culture increased as the concentration of acetate was increased to* 0.01 *M; higher concentrations of acetate resulted in lower yields of carotene. An acetate concentration of* 0.0075 *M was chosen for further work.*

AGITATION

In many experiments it was desired to add additional nutrients and tracers to growing cultures. It became necessary, therefore, to determine if gentle agitation had any effect on the synthesis of carotene.

Agitation was begun on the third day of incubation and repeated daily until the penultimate day of incubation. The tote boxes which contained 20 cultures were picked up and swirled gently for 5 seconds in the first experiment. The data for the production of mycelium and carotene are given in Figure 11.

In another experiment the flasks were gently swirled on the fourth and fifth day only. One μc. of acetate-1-C^{14} was added to some of the flasks on the fourth day. The data for the determinations on the seventh

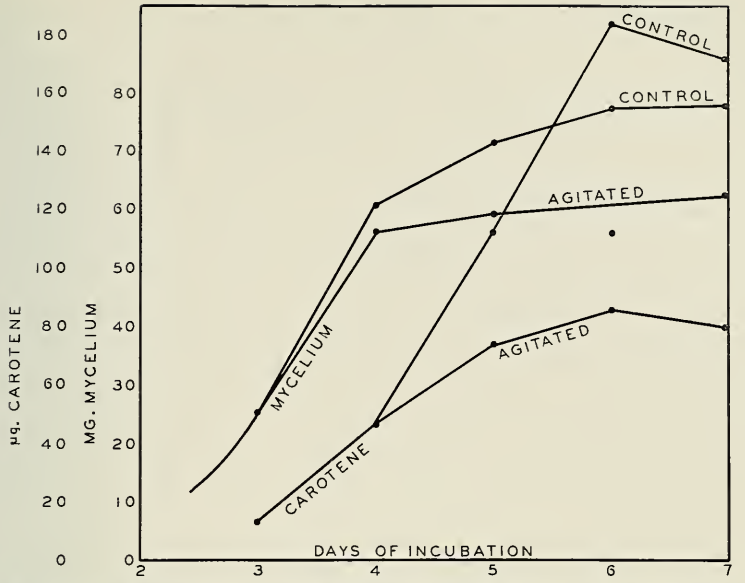

FIGURE 11. The effect of gentle agitation once a day on the production of mycelium and carotene.

day of incubation for control and agitated cultures, respectively, were as follows: dry weight, 68.1, 63.8 mg.; pH, 2.48, 2.71; residual glucose, 17, 29 mg.; and carotene, 153, 135 μg. The agitated cultures utilized acetate slightly slower than the control cultures. The initial C.P.M./flask was 654,000; 24 hours later the C.P.M. of the control flasks was 22,250 while the C.P.M. of the agitated flasks was 43,250. These experiments showed that agitation depressed growth and carotenogenesis and slowed the utilization of glucose and acetate.

It was found that the flasks contained in a tote box could be gently tilted without reducing the yield of carotene. This was done by slowly raising one end of the tote box about three inches and slowly lowering it. This procedure is referred to as "tilting" elsewhere in the Bulletin.

Agitation of the cultures of P. blakesleeanus after the mats have formed resulted in a decreased yield of carotene. Agitation also resulted in slower growth and a decreased rate of glucose and acetate utilization. "Tilting" did not appreciably affect either growth or carotenogenesis.

CONCENTRATION OF NITROGEN

Goodwin and Wilmer (1952) reported that the presence of nitrogen in the medium was not essential for carotenogenesis. Preformed mycelial mats were used in these experiments. More carotene was synthesized

on a glucose-salts medium than on a complete medium containing asparagine. Nitrogen is of course essential for the formation of mycelium.

The concentration of ammonium sulfate in the basal medium was varied between 0.25 and 2.0 g./l. The data for the determinations made on the seventh day of incubation are presented in Figure 12. Concentrations of ammonium sulfate greater than 1 g./l. had little effect on the yield of mycelium or the production of carotene. The utilization of glucose was more complete when 2.0 rather than 1.0 g./l. of ammonium sulfate was used. The pH of the media was not affected by increasing the ammonium sulfate concentration from 1.0 to 2.0 g./l. Sporangiophores were produced in the media containing 0.25 and 0.5 g./l. of ammonium sulfate by the end of 5 days; they were not formed by cultures which received 1.0 g./l. or more of ammonium sulfate. From other experiments it is known that about four-fifths of the nitrogen was utilized when the medium contained 1.0 g./l. of ammonium sulfate (Lilly *et al.* 1958).

Concentrations of ammonium sulfate in excess of 1 *g./l. had little effect on the production of mycelium and carotene. Concentrations less than* 1 *g./l. resulted in decreased yields of mycelium and carotene.*

DECREASE IN CAROTENE CONTENT IN OLD MYCELIUM

The carotenoids are unstable compounds. A decrease in the carotene content of old cultures of *Phycomyces blakesleeanus* was noted by Garton *et al.* (1951). Grob (1953) reported that the carotene content of *Mucor hiemalis* mycelium decreased between the twentieth and fortieth day of incubation. The loss of β-carotene during this 20-day period was 69 per cent. New carotenoids were formed during this period; mutachrome was identified among the carotenoids isolated from 40-day-old mycelium. Grob attributed the decrease in carotene content to oxidation.

We have observed that the carotene content decreases as the cultures age. In general, the carotene content does not increase after all of the glucose is utilized. The effect of adding two carbon sources to 10-day-old cultures on the loss of carotene was determined. One hundred and twenty-five mg. of glucose (50 mg. C/culture) was added to one set of cultures (carotene content 150 μg./culture), while the same weight of potassium acetate (31 mg. C/culture) was added to a second group of cultures (carotene content 128 μg./culture). Harvests were made at 14, 18, and 37 days. Both compounds decreased the rate at which the carotene content of the mycelium decreased. The data for the control and glucose-treated culture after 4, 8, and 27 days were 147 vs. 153; 90 vs. 122; and 18 vs. 69 μg./culture, respectively. The corresponding data for the control and acetate-treated cultures after 4, 8, and 27 days

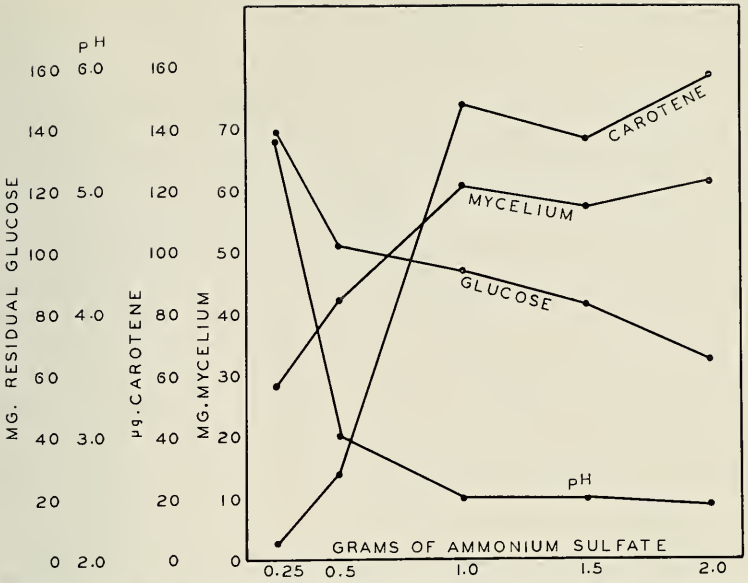

FIGURE 12. The effects of varying the concentration of ammonium sulfate in the basal medium on the production of mycelium, the pH values of the culture filtrates, the utilization of glucose, and the accumulation of carotene. The data are for 7-day-old cultures.

were 116 vs. 126; 81 vs. 107; and 13 vs. 84 μg./culture, respectively. The addition of either glucose or potassium acetate to 10-day-old cultures decreased the rate at which carotene was destroyed.

The carotene content reaches a maximum about the time that the glucose is completely utilized, thereafter the carotene content of the mycelium decreases. The addition of glucose or potassium acetate to 10-day-old cultures decreases the rate at which carotene disappears from the mycelium.

LIGHT

A number of fungi have been reported to accumulate more carotene in light than in darkness. *Penicillium sclerotiorum* (Mase *et al.* 1957), *Neurospora crassa* (Zalokar 1954), and *Phycomyces blakesleeanus* (Garton *et al.* 1951 and Chichester *et al.* 1954) synthesize more carotene when the cultures are illuminated than in darkness.

P. blakesleeanus in our experiments produced more carotene when the cultures received 50 foot-candles from white fluorescent lamps for 12 hours daily than was produced by cultures which received from 1 to 2 foot-candles, or by those kept in darkness (Figure 13). The production of mycelium, the utilization of glucose, and the changes in the pH values of the media were essentially the same for these 3 conditions

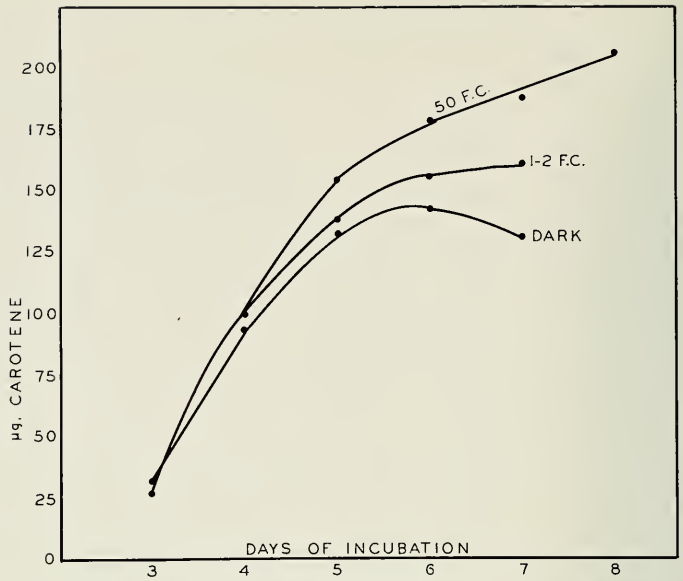

FIGURE 13. The effects of 3 levels of illumination on the accumulation of carotene.

(Lilly *et al.* 1957). The intensity of light had little effect on carotene synthesis until after the fourth day.

The effects of continuous vs. 12-hour illumination (50 to 60 foot-candles) were compared. There was no appreciable difference in growth, glucose utilization, or pH values of the medium between 3 and 9 days between cultures kept under these 2 conditions. Slightly more carotene was produced by the cultures illuminated continuously than by those which were illuminated 12 hours daily. The yields of carotene on the eighth day were: continuous light, 204 µg./flask; 12 hour, 189 µg./flask.

An experiment was made in which cultures were exposed to 950 foot-candles for 12 hours. The temperature of the medium when the lights were on was 25° C.; the temperature during the 12 hours of darkness was 20° C. As much carotene was produced by these cultures by the fifth day as by control cultures kept at 25° C. and illuminated continuously (50 to 60 foot-candles). The carotene content of cultures receiving 950 foot-candles did not increase appreciably after the fifth day of incubation.

Light had little effect on the production of carotene until most of the mycelium was formed; thereafter the carotene content of cultures exposed to light was greater than that of cultures kept in darkness. Continuous illumination (50 to 60 foot-candles) was slightly better than 12-hour illumination for the production of carotene.

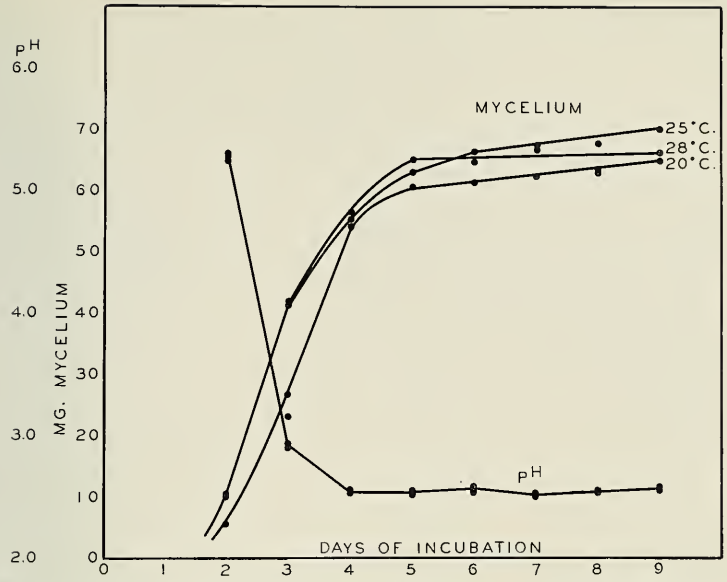

FIGURE 14. The effects of 3 temperatures of incubation on the production of mycelium and the changes in pH values of the culture medium.

TEMPERATURE

The optimum temperature for carotenogenesis by *Phycomyces blakesleeanus* is 25° C. (Friend and Goodwin 1954). It is not known whether this is true of all isolates of this species.

The effects of temperature were studied in a number of experiments. The data obtained in one experiment at 20°, 25°, and 28° C. are presented in Figures 14 and 15. The pH values (Figure 14) were essentially the same for the 3 temperatures throughout 9 days of incubation. Slightly more mycelium was produced at 25° than at 20° or 28° C. The initial rate of growth at 20° C. was less than at the higher temperatures (Figure 14). Glucose was utilized more rapidly at 25° than at 20° or 28° C. (Figure 15). Sporangiophores from 2 to 3 inches long were produced on the fifth day of incubation by the cultures kept at 20° C. The pH of the medium was about 2.5 at this time. Only a few short sporangiophores were produced at 25° or 28° C. Normal long sporangiophores were produced at 25° only when the pH value of the medium remained above 3.5 (p. 16).

Twice as much carotene was produced by the ninth day of incubation at 25° than at 20° C. (Figure 15). The rate of carotene production at 25° and 28° C. was approximately the same for the first 6 days of incubation; a slow accumulation of carotene continued in the 25° C.

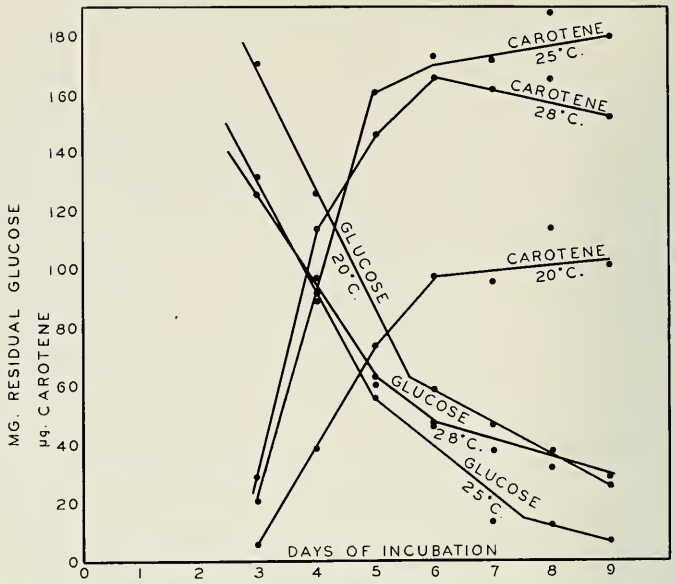

FIGURE 15. The effects of 3 temperatures of incubation on the utilization of glucose and the accumulation of carotene.

cultures, whereas the carotene content of the 28° C. cultures decreased slightly after the sixth day of incubation.

The importance of rigid temperature control was demonstrated in experiments when the temperature controls failed. A considerable decrease in carotene yield was observed at 24° C. The effect of low temperatures (23° to 24° C.) was to greatly reduce the carotene yield if low temperatures occurred during the first 4 days of incubation. Sporangiophores formed abundantly at 23° to 24° C. Increasing the temperature to 25° to 26° C. on the third or fourth day of incubation did not overcome the deleterious effect of earlier low temperature on carotene production.

In order to obtain high yields of carotene with this isolate of P. blakesleeanus, it is necessary to maintain the temperature between 25° *and* 26° *C. This temperature may be favorable for carotenogenesis because it inhibits asexual sporulation.*

INITIAL pH

In the earlier work the pH value of the medium at the time of inoculation (initial pH) appeared to have considerable effect on the amount of carotene synthesized. Initial pH values of 4.12, 5.20, 5.80 and 6.74 resulted in the following yields of carotene for these media: 52, 175, 181, and 118 μg./flask (9 days). The corresponding weights of mycelium were 27.1, 57.7, 69.4, and 69.9 mg.

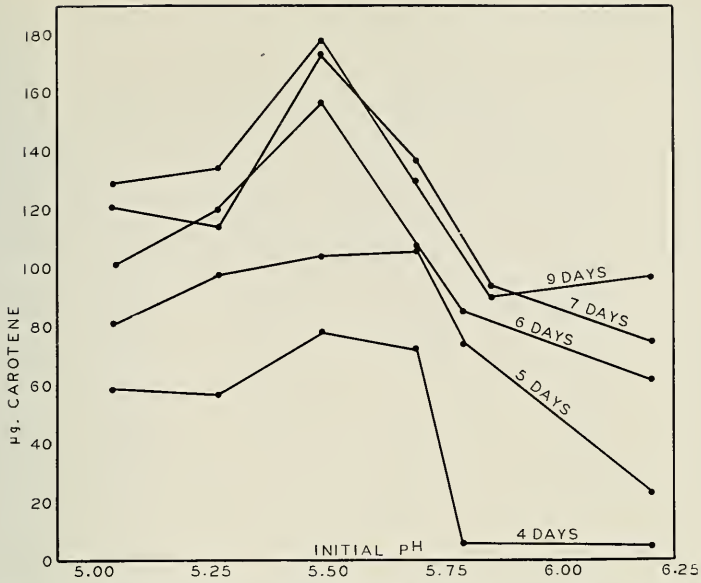

FIGURE 16. The effect of initial pH on the accumulation of carotene at 23° C. and 12 hours light (100 foot-candles).

It was found that the temperature of incubation influenced the yield of carotene in the pH range of 5 to 6. When the cultures were incubated at 23° C. an initial pH value of about 5.5 resulted in the highest yield of carotene (Figure 16). Cultures incubated at 25° C. were less sensitive to variation in initial pH. Little difference in yields between pH 5.25 and 5.75 were noted (Figure 17). The same lot of basal medium was used in both of these experiments. Data for the production of mycelium and changes in pH values for media having initial pH values of 5.06, 5.70, and 6.20 are given in Figure 18.

Every experiment with an organism is unique. The results are affected by a host of variables some of which are not recognized. The data for the production of mycelium and carotene, pH values, and residual glucose for 9 consecutive experiments are presented in Table 1. The known variable in these experiments was the initial pH which ranged from 5.50 to 5.95. The cultures were exposed to continuous light (50 to 60 foot-candles) and the temperature of incubation was 25° to 26° C. The micro element concentration was 2 ×.

It may be noted first that growth and carotenogenesis were slower in the media having the higher pH values (compare with Figures 17 and 18). If the experiments in which the initial pH values were between 5.50 and 5.67 are considered as a sub-group only one experiment (No. 3)

TABLE 1. DATA FROM SERIAL EXPERIMENTS ON THE PRODUCTION OF MYCELIUM AND CAROTENE, pH VALUES, AND RESIDUAL GLUCOSE BY *Phycomyces blakesleeanus*, ISOLATE 104, 2 × MICRO ELEMENTS, 25° TO 26° C., AND CONTINUOUS LIGHT (50 TO 60 FOOT-CANDLES)

EXPERIMENT NO.	MYCELIUM mg./FLASK	pH		RESIDUAL GLUCOSE mg./FLASK	CAROTENE μg./FLASK
		INITIAL	FINAL		
		3rd Day Harvest			
1	54.4	5.50	2.55	119	60
2	49.5	5.50	2.40	173	48
3	29.5	5.50	3.76	206	12
4	48.2	5.60	2.83	156	24
5	48.0	5.67	2.65	176	36
6	22.2	5.75	4.80	231	6
7	41.5	5.90	3.13	—	20
8	15.4	5.90	4.75	245	7
9	21.1	5.95	4.55	162	6
Means	**36.6**	**5.70**	**3.49**	**184**	**24**
		4th Day Harvest			
1	64.2	5.50	2.53	85	132
2	64.8	5.50	2.54	128	134
3	54.1	5.50	2.69	122	65
4	69.8	5.60	2.58	102	113
5	63.8	5.67	2.62	138	85
6	56.1	5.75	2.65	104	67
7	66.7	5.90	2.55	—	90
8	37.5	5.90	3.20	183	22
9	53.5	5.95	2.60	140	38
Means	**58.9**	**5.70**	**2.66**	**125**	**83**
		5th Day Harvest			
1	67.0	5.50	2.55	63	172
2	69.8	5.50	2.50	78	182
3	65.1	5.50	2.55	85	141
4	74.1	5.60	2.55	92	156
5	69.8	5.67	2.61	70	146
6	73.9	5.75	2.48	54	182
7	74.1	5.90	2.51	—	158
8	67.0	5.90	2.48	77	109
9	67.3	5.95	2.55	106	115
Means	**69.8**	**5.70**	**2.53**	**98**	**151**
		6th Day Harvest			
1	74.3	5.50	2.51	57	168
2	73.7	5.50	2.58	54	191
3	71.3	5.50	2.45	55	158
4	75.0	5.60	2.58	32	191
5	73.8	5.67	2.58	55	217
6	74.2	5.75	2.40	51	216
7	78.1	5.90	2.50	—	208
8	69.8	5.90	2.55	55	168
9	73.9	5.95	2.30	62	147
Means	**74.9**	**5.70**	**2.49**	**53**	**185**

(Table 1 Continued on Page 29)

7th Day Harvest

1	76.5	5.50	2.50	58	164
2	76.7	5.50	2.60	22	188
3	68.8	5.50	2.35	29	194
4	82.3	5.60	2.55	23	193
5	74.0	5.67	2.55	36	218
7	81.1	5.90	2.50	—	222
8	73.6	5.90	2.55	51	179
9	77.9	5.95	2.50	33	179
Means	**76.4**	**5.72**	**2.51**	**36**	**192**

8th Day Harvest

1	73.9	5.50	2.55	38	180
2	79.9	5.50	2.55	—	209
3	74.4	5.50	2.40	14	202
7	76.2	5.90	2.55	—	232
8	75.4	5.90	2.50	47	213
9	78.9	5.95	2.45	14	203
Means	**76.4**	**5.71**	**2.50**	**28**	**207**

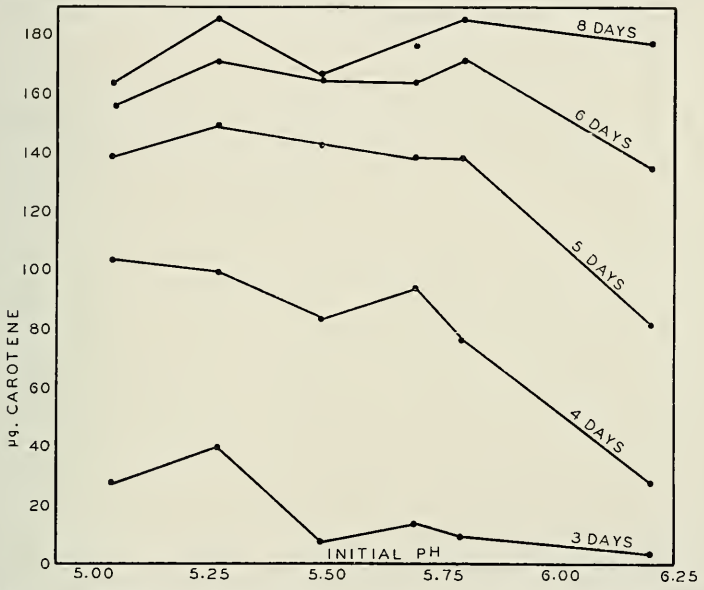

FIGURE 17. The effect of initial pH on the accumulation of carotene at 25° C. and continuous light (50 to 60 foot-candles).

showed great deviation from the means. In the other sub-group where the initial pH values varied between 5.75 and 5.95 one experiment (No. 7) showed pronounced deviation from the means. It is possible that the initial pH values in experiments 3 and 7 were determined incorrectly but it also is possible that some other factor is responsible.

From the standpoint of producing radioactive β-carotene it was considered essential to use cultures which were in the phase of active

carotenogenesis (as determined by sub samples). Acetate-C^{14} was usually not added until the fourth or fifth day of incubation. Cultures which were found to weigh less than 40 mg. on the third day of incubation, or which had produced less than 90 μg. of carotene by the fourth day were ordinarily not used for the production of radioactive β-carotene.

The effects of initial pH values of the basal medium are more pronounced at 23° C. than at 25° to 26° C. It is considered that initial pH values between 5.50 and 5.65 are more suitable than higher or lower values.

EFFECTS OF INCREASING THE pH VALUES DURING GROWTH

High rates of carotene synthesis occurred only after the pH value of the culture medium decreased to 3 or less. In the experiments described below the pH values of the culture medium were increased during the phase of active carotenogenesis. This was done to study further the effect of pH on carotenogenesis.

In the first series of experiments various amounts of sodium carbonate were added to 4-day-old cultures when the pH of the medium was 2.75. Figure 19 shows the immediate and subsequent changes in the pH values of the culture media. The addition of 0.5, 0.75 and 1.0 ml. of N/10 sodium carbonate (2.7, 4.0, and 5.3 mg.) did not greatly affect growth. The data for the harvest on the eighth day were as follows: control, 70.5 mg.; 0.5 ml., 74.4 mg.; 0.75 ml., 72.4 mg.; and 1.0 ml., 70.8 mg. The weight of cultures receiving 2.0 ml. of N/10 sodium carbonate was 80.9 mg. after 8 days. The corresponding values for residual glucose (8 days) for the control, 0.5, 0.75, 1.0 and 2.0 ml. N/10 sodium carbonate were 29, 26, 22, 22, and 6 mg, respectively. Sporangiophores from 0.5 to 1 inch long were produced by cultures receiving 0.75 and 1.0 ml. N/10 sodium carbonate, while sporangiophores 2 to 3 inches long were produced by cultures receiving 2 ml. N/10 sodium carbonate.

The effect of partially neutralizing culture media on carotenogenesis is shown in Figure 20. The addition of as little as 0.5 ml. N/10 sodium carbonate resulted in a decrease in the amount of carotene present. A little carotene appeared to be synthesized after the addition of 0.75 ml. N/10 sodium carbonate. The carotene concentration of cultures receiving 1.0 ml. N/10 sodium carbonate did not increase after its addition, while the concentration of carotene in the mycelium decreased when 2.0 ml. N/10 sodium carbonate was added. The results showed that increasing the pH values on the fourth day of incubation by adding sodium carbonate decreased the rate of carotene synthesis. This effect was proportional to the increase in pH values when 0.5 to 1.0 ml. N/10

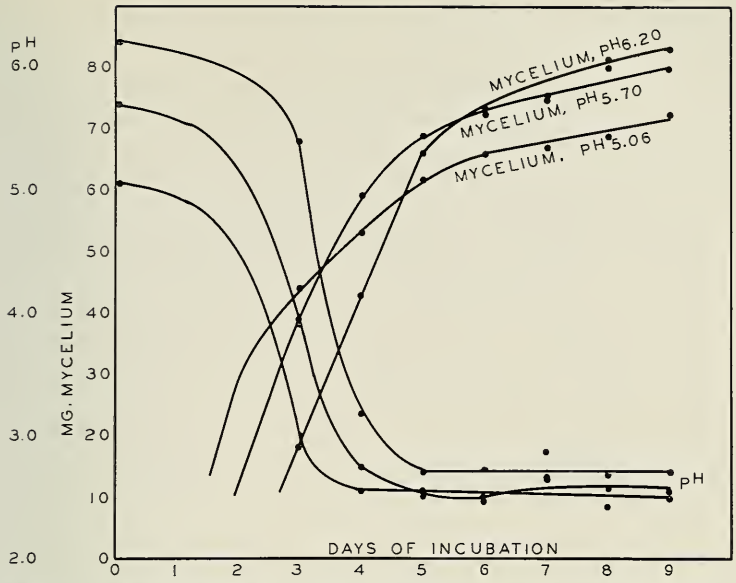

FIGURE 18. The effects of 3 initial pH values on the production of mycelium and changes in pH values of the culture media.

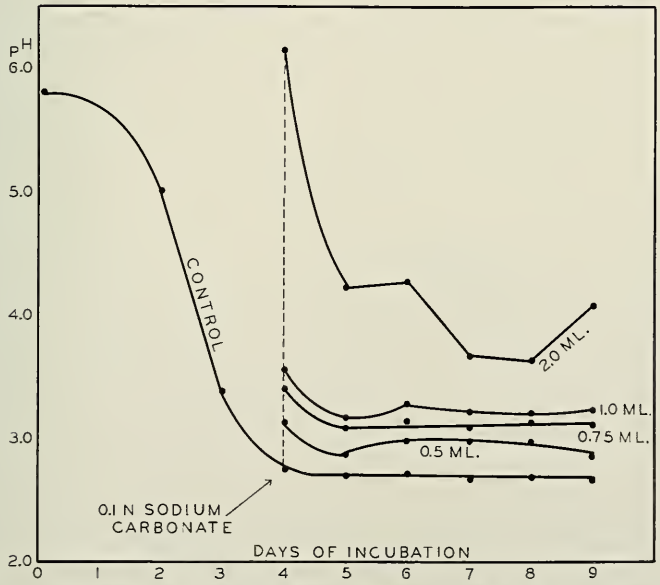

FIGURE 19. The effects of adding various amounts of sodium carbonate on the immediate and subsequent pH changes of the culture media.

sodium carbonate was added. The addition of 2.0 ml. N/10 sodium carbonate/culture resulted in failure of the cultures to accumulate more carotene than was present at the time the sodium carbonate was added. Presumably, this was due to conditions becoming favorable for growth and sporulation.

The second method of increasing the pH value of 4-day-old cultures was to add potassium acetate. Four series of cultures were treated with 3, 6, 12, and 24 mg. potassium acetate; the immediate and subsequent changes in pH values of the culture media are shown in Figure 21.

Both sodium carbonate and potassium acetate were effective in increasing the pH of the culture media as may be seen by comparing Figures 19 and 21. The effects of the addition of potassium acetate on the fourth day on the production of mycelium are shown in Figure 22. The addition of 3 and 6 mg. of potassium acetate resulted in a slight, but probably not significant, increase in carotene content (Figure 23). However, the addition of 12 and 24 mg. of potassium acetate on the fourth day of incubation resulted in the accumulation of less carotene than in the control cultures. The addition of potassium acetate resulted in slower utilization of glucose, except when 24 mg. of potassium acetate was added. Glucose utilization was more rapid and complete in these cultures than in the control cultures; presumably this was due to the more favorable pH value for growth.

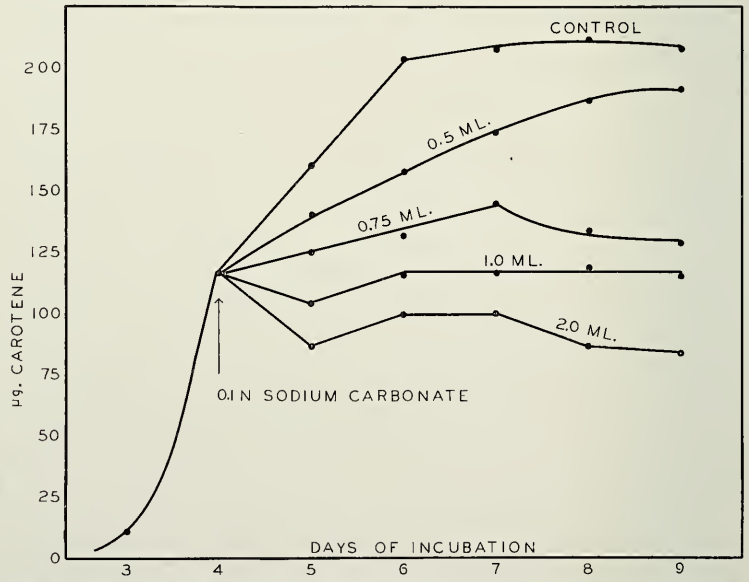

FIGURE 20. The effects of partially neutralizing the culture media of 4-day-old cultures with sodium carbonate on subsequent production of carotene.

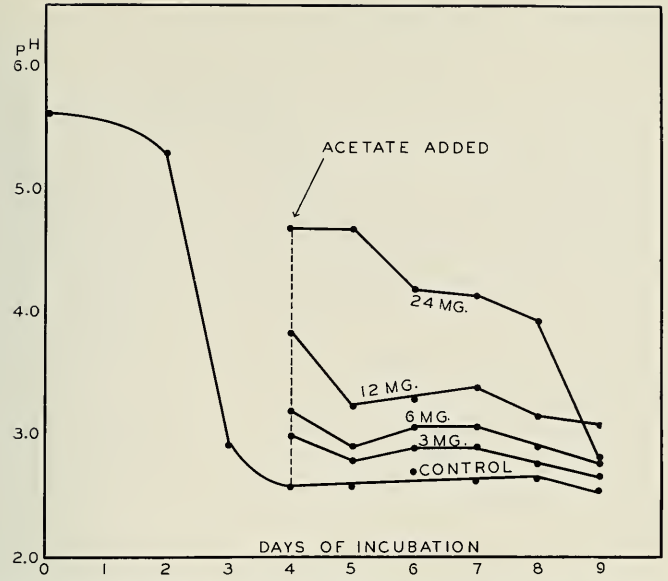

FIGURE 21. The effects of adding various amounts of potassium acetate on the immediate and subsequent pH changes of the culture media.

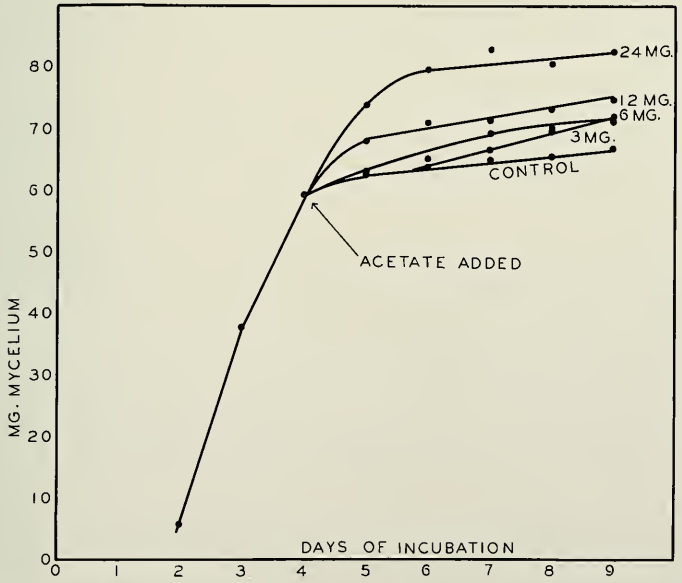

FIGURE 22. The effects of adding various amounts of potassium acetate to 4-day-old cultures on the subsequent production of mycelium.

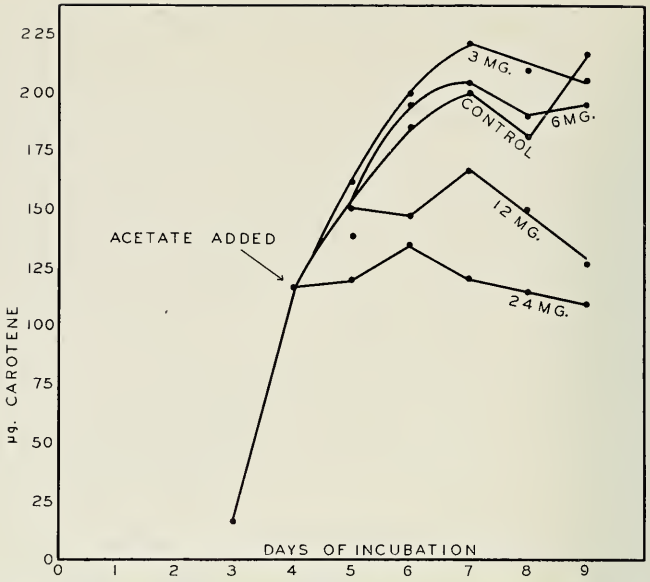

FIGURE 23. The effects of adding various amounts of potassium acetate to 4-day-old cultures on the subsequent production of carotene.

The results obtained in these two series of experiments were similar when the amounts of sodium carbonate (1 to 2 ml.) and potassium acetate (12 to 24 mg.) were sufficient to increase the pH of the culture media above 3.15 (Figures 19 and 21). These concentrations resulted in partial or complete inhibition of carotene synthesis (Figures 20 and 23). The low concentrations of sodium carbonate (0.5 and 0.75 ml. N/10) inhibited carotenogenesis (Figure 20), while low concentrations of potassium acetate (3 or 6 mg.) slightly increased carotenogenesis (Figure 23). The effects of these low concentrations of these compounds in raising the pH of the culture media were about equal (Figures 19 and 21).

Sodium carbonate and potassium acetate have the same effects (increased growth and inhibition of carotenogenesis) provided that the concentrations used are sufficient to maintain the pH levels above 3.2. On the other hand, the effects of low concentrations of carbonate and acetate are different even when the pH levels are raised to the same value by these compounds. Low concentrations of sodium carbonate inhibited carotenogenesis while low concentrations of potassium acetate increased carotenogenesis. In neither instance does the compound added persist; it would be expected that carbonate added to acidic media would be decomposed immediately. Separate experiments showed that most of

the added acetate was utilized within 6 hours and essentially all by the end of 24 hours, yet the effects of these compounds persisted for days.[1]

Partial neutralization of 4-day-old culture media by the addition of either sodium carbonate or potassium acetate reduced the yield of carotene when the pH values were increased to 3.2 or more. Low concentrations of sodium carbonate and potassium acetate which did not increase the pH values of the culture media to 3.0 had different effects: low concentrations of sodium carbonate decreased the yields of carotene; low concentrations of potassium acetate increased yields of carotene.

SUPPLEMENTARY NUTRITION

The medium replacement technique is frequently used in the study of metabolic processes in fungi. In this process the old medium is withdrawn and is replaced by another. Carotenogenesis by *Phycomyces blakesleeanus* is adversely affected when the mats are disturbed. In order to avoid agitating the mats the nutrients were added in 1 ml. of sterile solutions by means of pipettes; care was taken to flow the solutions down the sides of the flasks.

Glucose

In one experiment 7.5 (3 mg. C) and 15 (6 mg. C) mg. of glucose was added daily from the fifth to the penultimate day of incubation. On the ninth day of incubation the dry weights of mycelium from the control, 7.5, and 15 mg. cultures were 75.1, 78.4, and 80.1 mg., respectively; on the twelfth day the corresponding weights were 64.5, 81.3, and 81.7. These additions of glucose were without effect on the pH values of the culture media, 2.62 to 2.65 from the ninth to twelfth day. Higher levels of glucose were maintained in the culture media when glucose was added daily. The data for residual glucose and carotene content are presented in Figure 24.

On the basis of this evidence it was concluded that carotene synthesis ceases about the time all of the glucose is utilized. By adding glucose daily carotenogenesis may be prolonged. These results suggest that carotene is formed from glucose soon after it enters the cells, and not from cellular constituents.

The period during which carotene is synthesized is prolonged by the daily addition of 7.5 or 15 mg. of glucose.

Yeast Extract

Friend *et al.* (1955) reported some effects of yeast extract (1 g./l.) on

[1]A study of the effects of sodium chloride is under way. Under certain conditions, the addition of 0.5 g./l. of sodium chloride to the basal medium has reduced or delayed spore germination. The early yields of carotene have been reduced.

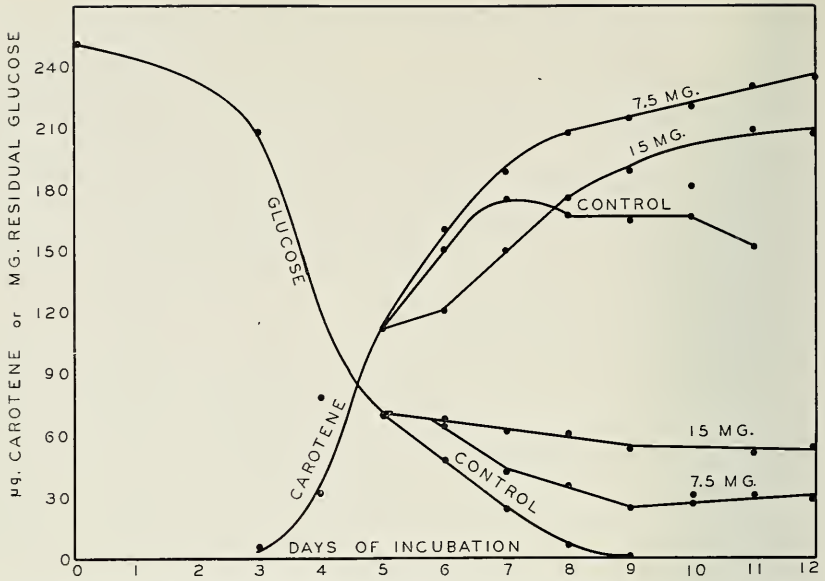

FIGURE 24. The effects of the daily addition of two concentrations of glucose on carotenogenesis and the level of residual glucose remaining in the medium.

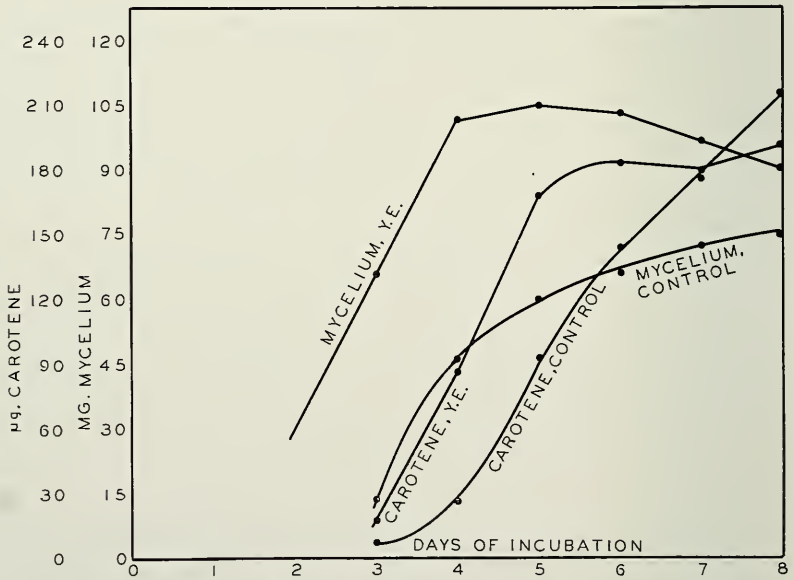

FIGURE 25. The effects of adding yeast extract (1 g./l.) to the basal medium on the production of mycelium and carotene.

growth and carotenogenesis by *Phycomyces blakesleeanus*. Media of various composition were used. The addition of yeast extract resulted in increased production of mycelium in most instances; carotene production was increased on certain media and decreased in others. The pH values of the culture media were not reported; however, from the composition of the media used they were probably 7 or higher. Chichester *et al.* (1954) used a glucose-yeast autolysate medium to which uniformly labeled radioactive fructose was added. From the radioactivity of the isolated β-carotene and radioactivity of the fructose added it was concluded that 61 per cent of the carbon in the sample of β-carotene was derived from the non-carbonhydrate (yeast autolysate) part of the medium. The non-sugar carbon content of the medium was 9 per cent of the total carbon.

In some experiments, the basal medium was modified by adding 1 g./l. of yeast extract. The initial rates of growth and carotenogenesis were greater in the modified than in the basal medium (Figure 25). More mycelium was produced in the modified than the basal medium. The effect of this concentration of yeast extract on growth is about the same as 0.02 *M* acetate (Figure 2). Both substances caused rapid utilization of glucose. This concentration of yeast extract had little effect on the pH level of the culture medium, the pH values of the modified and basal media on the fifth day were 2.66 and 2.63, respectively. Carotene synthesis between the third and fifth day was rapid on the modified medium, but more carotene was synthesized eventually on the basal medium. Very little carotene was synthesized when the basal medium was 0.02 *M* in acetate (Figure 5). Whether the good carotene yield in the modified yeast extract basal medium was due to the low pH values of the culture medium, or to carotene precursors present in the yeast extract is not known.

The effects of reducing the concentration of yeast extract to 50 mg./l. were also investigated. The yeast extract was added to one set of cultures at the time of inoculation (0 days) and to another set of the fourth day. Yeast extract resulted in increased production of mycelium, especially during the early days of incubation, and a more rapid decrease in the pH values of the culture media than in the control cultures (Figure 26). The effects on carotenogenesis and glucose utilization were less marked than obtained when 1 g./l. of yeast extract was used (Figure 27).

The effectiveness of yeast extract in increasing early growth and carotenogenesis is greater when it is added at the time of inoculation than after four days. Yeast extract added to 4-day-old cultures increased the utilization of glucose, but not as markedly as when it was added at the

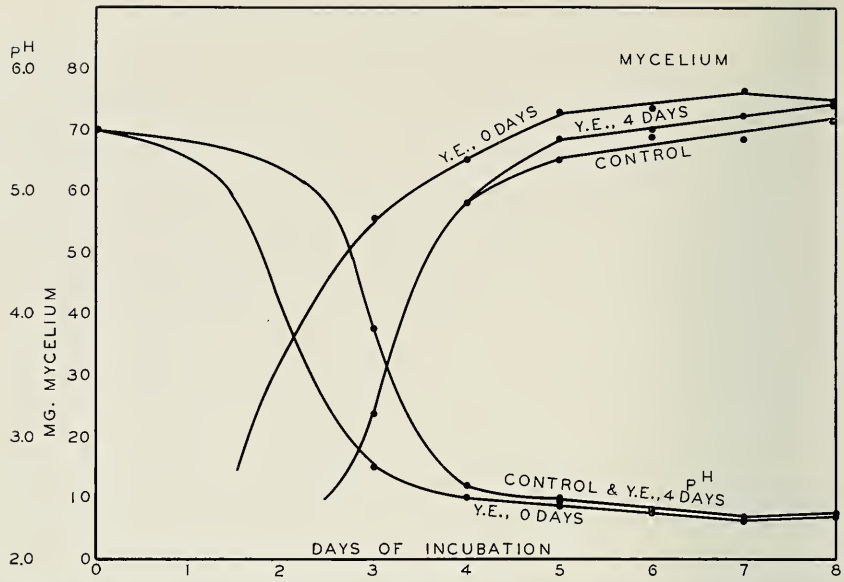

FIGURE 26. The effect of adding yeast extract (50 mg./l.) to the basal medium on the production of mycelium and changes in pH values.

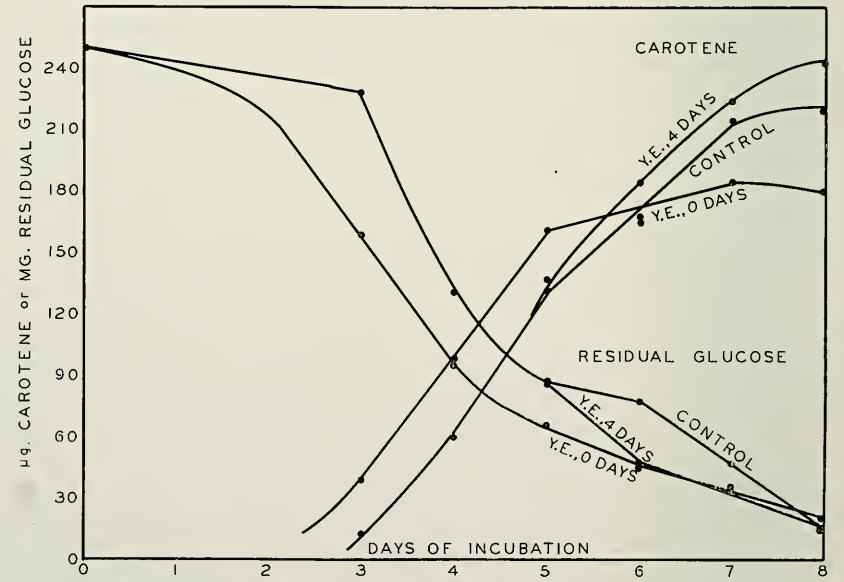

FIGURE 27. The effects of adding yeast extract (50 mg./l.) to the basal medium on the production of carotene and the utilization of glucose.

time of inoculation. It would appear that yeast extract may contain a compound which increases carotene synthesis.

The addition of yeast extract to the basal medium results in more rapid growth and carotenogenesis than in the basal medium.

Acetic Acid

When 12 and 24 mg. of potassium acetate were added to 4-day-old cultures, the pH values increased initially from 2.60 to 3.80 and 4.70 (Figure 21) and reduced yields of carotene (Figure 23) were obtained. The reduced yields of carotene were attributed to the increased pH values of the medium. Since labeled acetate was used for the biosynthesis of radioactive β-carotene, a series of experiments were made to determine the effect of adding acetic acid to growing cultures. It proved more convenient to use acidified potassium acetate than acetic acid. Potassium acetate solutions of the desired concentration were acidified with hydrochloric acid to a pH value of 2.75. This treatment converted about 99 per cent of the potassium acetate into acetic acid and potassium chloride.

Two concentrations of acidified potassium acetate, 12.2 mg. (3 mg. C) and 24.4 mg. (6 mg. C) were added on the fifth day of incubation, and were continued daily until the penultimate day of the experiment. The temperature of incubation was 25° C. and the cultures were illuminated 12 hours daily (100 foot-candles). In neither instance did the pH values of the culture media differ essentially from those of the control media. Cultures which received the daily addition of 12.2 mg. of acidified potassium acetate made slightly better growth (79.8 mg., 10 days) than the control cultures (72.1 mg., 10 days), but the dry weights of mycelium of cultures receiving 24.4 mg. of acidified potassium acetate did not increase from the sixth to the tenth day.

The data for the production of carotene and utilization of glucose are given in Figure 28. Carotene production was increased by the daily addition of 12.2 mg. of acidified potassium acetate, but decreased when the concentration was doubled. The daily addition of 12.2 mg. of acidified potassium acetate resulted in a decrease in the amount of glucose utilized. The sparing effect of this quantity of acidified potassium acetate on glucose utilization was observed in all subsequent experiments. If these results are compared with those reported previously (Figure 23) it may be concluded that the effect on carotenogenesis of adding acetate depends upon the pH value of the medium.

The cultures which received daily 24.4 mg. of acidified potassium acetate failed to make additional growth; glucose utilization ceased, and the carotene content of the mycelium decreased after the sixth day. It was concluded that the concentration of unionized acetic acid was great

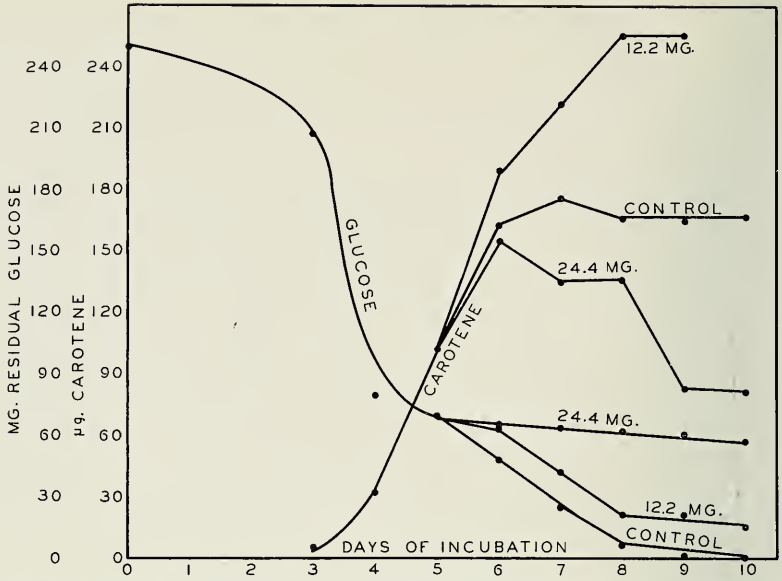

FIGURE 28. The effects of adding 12.2 and 24.4 mg. of acidified potassium acetate to 5-day-old cultures on the production of carotene and the utilization of glucose.

enough to be toxic. The addition of 24 mg. of potassium acetate in a previous experiment (Figure 22) was not toxic, presumably because the concentration of unionized acetic acid was about half that in this experiment[1].

Many additional experiments were made in which acidified potassium acetate was added to growing cultures. It was thought possible that more acetate-carbon would be incorporated into carotene under these conditions than when C^{14}-labeled acetate alone was added since the addition of 12.2 mg. of acidified potassium acetate decreased the amount of glucose utilized. In general, less carotene was produced in cultures to which acidified potassium acetate was added if the temperature of incubation was less than 24° C. This conclusion is based on experiments in which failure of the temperature controls occurred. In some experiments, it was noted that bleached areas of mycelium resulted from excessive local concentrations of acidified potassium acetate. The obvious remedy for this situation was to mix the added acidified potassium acetate with the culture medium. This mixing has to be done with care

[1]The concentrations of unionized acetic acid/flask were calculated by using the Henderson-Hasselbalch equation: $pH = pKa + \log [salt]/[acid]$ and the concentrations of acetate used. The concentrations of unionized acid were for 12.2 (pH 2.6), 24.4 (pH 2.6), and 24 (pH 4.70) mg. of potassium acetate; 7.45, 14.9, and 7.85 mg., respectively.

otherwise the agitation will disturb the mats sufficiently to reduce the yield of carotene. The technique for mixing has already been described.

As much as 12.2 *mg. of acidified potassium acetate may be added daily to* 4*-day and older cultures of Phycomyces blakesleeanus. The acetate is utilized. Glucose utilization is less when acetate is added. As much or more carotene is produced by cultures receiving added acidified potassium acetate as by control cultures. The mixing of the acidified potassium acetate with the culture medium must be carefully done so as to disturb the mats as little as possible.*

SIZE OF INOCULUM

The usual inoculum was one drop of spore suspension (about 100,000 spores). To determine whether this was adequate the following experiment was made. Four levels of inoculum were used: 1,000,000, 100,000, 10,000, and 1,000 spores. The same lot of spores was used to prepare the the dilutions, and the same lot of basal medium was used. The cultures were incubated at 25° C. and received 50 to 60 foot-candles of light 24 hours daily.

As expected, the differences among these cultures was significant during the early days of incubation. As the time of incubation was prolonged these differences became smaller and smaller. Within narrow limits, the cultures receiving 1,000,000, 100,000, and 10,000 spores were equivalent after eight days of incubation. The yield of carotene from cultures receiving 1,000 spores was less than the other cultures. Some of the data are presented in Table 2.

For rapid production of carotene not less than 100,000 *spores should be used per flask.*

MIXED + AND — CULTURES

In view of the enhanced production of carotene by mixed + and — cultures of *Choanephora cucurbitarum* (Barnett *et al.* 1956) and various mixed cultures of other species of the Choanephoracea (Hesseltine and Anderson 1957), a study of carotene production by mixed — (104) and + (103) isolates of *Phycomyces blakesleeanus* was undertaken. These results (Lilly *et al.* 1957) may be summarized as follows: mixed + and — cultures of *P. blakesleeanus* under the environmental conditions used (different light intensity and temperature) produced about as much carotene as the — cultures alone. Mixed + and — cultures which received 50 foot-candles 12 hours daily and which were incubated at 25° C. produced slightly more carotene than either of the isolates under these conditions. *P. blakesleeanus* does not produce zygospores in liquid medium under these conditions but *C. cucurbitarum* does.

TABLE 2. EFFECT OF NUMBER OF SPORES USED AS INOCULUM ON THE PRODUCTION OF MYCELIUM, YIELD OF CAROTENE, AND THE UTILIZATION OF GLUCOSE[1]

DAYS	MYCELIUM mg. NO. OF SPORES USED				CAROTENE μg/CULTURE NO. OF SPORES USED				RESIDUAL GLUCOSE mg. NO. OF SPORES USED			
	10^6	10^5	10^4	10^3	10^6	10^5	10^4	10^3	10^6	10^5	10^4	10^3
3	29.5	23.8	22.4	10.0	12	12	8	—	206	203	243	—
4	54.1	54.7	49.9	32.2	65	64	58	11	122	124	133	193
5	65.3	65.1	61.7	53.3	149	141	108	79	78	85	98	105
6	71.1	71.3	68.0	67.3	178	158	125	153	51	55	76	75
7	72.0	67.9	68.8	69.5	205	194	182	176	26	29	33	44
8	72.6	74.4	73.2	72.3	217	202	185	176	16	14	27	40
9	72.5	73.3	—	—	214	203	—	—	8	8	—	—
10	—	—	71.7	—	—	—	217	—	—	—	5	—
11	—	—	—	73.4	—	—	—	181	—	—	—	5

[1]From the fifth day on the pH values for the four conditions differed by less than 0.1 pH unit.

Mixed + and − cultures of P. blakesleeanus did not produce appreciably more carotene than the − isolate alone.

COMPARISON OF ISOLATES

Goodwin and Griffiths (1952) compared the carotene production of five isolates of *Phycomyces blakesleeanus* and one isolate of *P. nitens* on a glucose-asparagine medium and found a fourfold variation in yield (9 days). It is not surprising that our four isolates differed in ability to synthesize carotene on the basal medium. Comparisons which are restricted to a single basal medium may not be valid when other media are used. Schopfer *et al.* (1952) found two isolates of *P. blakesleeanus* to produce the same amount of carotene when cultured in a lactate-acetate-ammonium medium. When these two isolates were cultured on a glucose-asparagine medium one isolate produced five times as much carotene as the other. It is possible that the full potential ability of certain isolates to produce carotene may be overlooked if only one basal medium and one set of environmental conditions are used.

Only two isolates (103 and 104) were available during the early part of this work. Preliminary experiments indicated that isolate 104 produced more carotene than isolate 103 (Lilly *et al.* 1957). Data for the production of carotene by isolates 103 and 104 at 2 temperatures of incubation are given in Figure 29. Isolates 1036 and 1177 produced much less carotene than either 103 or 104. The maximum yield of carotene obtained with isolates 1036 and 1177 were 42 and 60 μg./flask (8 days).

The concentration of carotene found in the mycelium of various isolates varied as much as fourfold.

EFFECT OF FLUORIDE

A number of compounds have been reported to inhibit carotenogenesis in bacteria and fungi. Grob *et al.* (1956) reported fluoride to inhibit growth and carotenogenesis by *Mucor hiemalis* when the fungus was cultured on a glucose medium; neither of these processes were inhibited when acetate was used as the source of carbon. The inhibition on glucose medium was attributed to the inhibition of the enzyme enolase which catalyzes the transformation of 2-phosphoglyceric acid into phosphoenolpyruvic acid (fluoride-inhibited cultures produced less than half as much pyruvic acid as the control cultures).

Potassium fluoride (4.7 mg./flask) was added to 4-day-old cultures of *Phycomyces blakesleeanus*. One set of flasks received fluoride alone, the second set fluoride and potassium acetate (6 mg./flask), and the third set acetate alone. The results may be summarized as follows: more my-

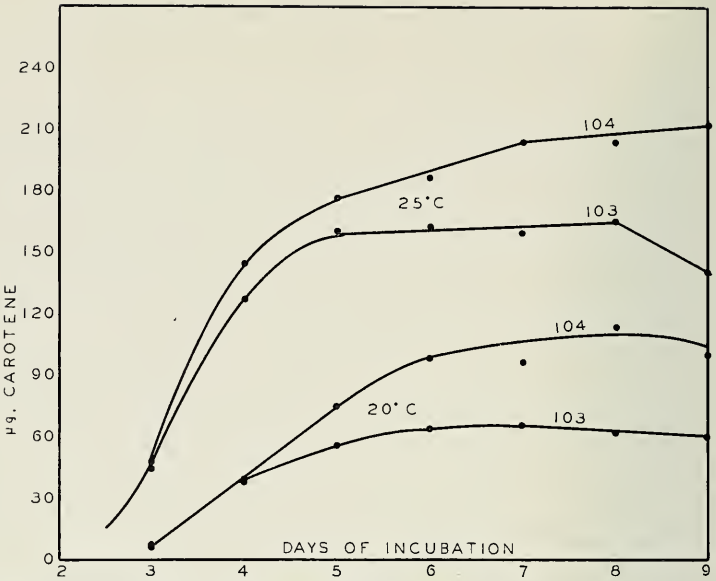

Figure 29. The production of carotene by isolates 103 and 104 at 20° and 25° C.

celium was produced by cultures to which acetate alone was added than by control cultures. The cultures which received fluoride, or fluoride and acetate did not increase in weight between the sixth and tenth day of incubation. Fluoride alone did not greatly affect the pH values of the culture filtrates. The addition of fluoride and acetate resulted in an increase in pH values (about 0.5 unit). Fluoride completely inhibited the utilization of glucose whether or not acetate was present. The synthesis of carotene was inhibited by the addition of fluoride, and under these conditions acetate did not overcome the inhibitory action of fluoride.

Fluoride under the conditions used in these experiments is a potent inhibitor of growth, carotenogenesis, and glucose utilization.

BIPHENYL

Villoutreix (1957) reported that biphenyl (20 to 200 mg./l.) had little effect on cell division of *Rhodotorula mucilaginosa* but inhibited carotenogenesis almost completely. Two concentrations of biphenyl (12.5 and 25 mg./l.) were used in our experiments with *Phycomyces blakesleeanus*. The basal medium was used, the temperature of incubation was 25° to 25.7°, and the cultures were illuminated continuously (50 to 60 foot-candles). These concentrations of biphenyl were without substantial effect on growth or carotenogenesis. The carotene content (μg./flask) of the control, 12.5, and 25 mg. biphenyl cultures were 210, 203, and 200 (6 days); and 223, 240, and 217 (10 days).

Growth and carotenogenesis of P. blakesleeanus were not appreciably affected by the addition of biphenyl (12.5 *or* 25 *mg./l.*).

β-IONONE

The terminal cyclic groups of the β-carotene molecule are β-ionone residues. Mackinney *et al.* (1952) found the addition of small amounts (0.5 to 5 μl.) of β-ionone to cultures of *Phycomyces blakesleeanus* growing on a glucose-yeast extract medium to result in a substantial increase in the β-carotene content of the mycelium.

Additional information on the β-ionone effect was reported by Mackinney *et al.* (1953). The greatest increase in carotene production by the β-ionone treated cultures occurred during the first 3 days of incubation. Chichester *et al.* (1954) showed that β-ionone increased the production of carotene by cultures of *P. blakesleeanus* kept in darkness, and that the effects of β-ionone and light are additive. Goodwin *et al.* (1956) reported a twofold increase in carotene production as the result of treating cultures of *P. blakesleeanus* with β-ionone; streptomycin was shown to overcome the effect of β-ionone. The use of β-ionone to increase the synthesis of carotene by fungi is not restricted to *P. blakesleeanus*. Anderson *et al.* (1958) found the addition of β-ionone to mixed + and − cultures of *Blakeslea trispora* to increase the yield of carotene. Chu (unpublished, this laboratory) found β-ionone to increase the amount of carotene synthesized by +, −, and mixed + and − cultures of *Choanephora cucurbitarum*.

Mackinney *et al.* (1952) suggested that β-ionone was incorporated directly into the β-carotene molecule by *P. blakesleeanus*. This attractive hypothesis was not substantiated when β-ionone-9-C^{14} (Engel *et al.* 1953) was used. The carotene synthesized under these conditions was not radioactive, hence the intact β-ionone molecule was not incorporated into β-carotene.

The emphasis in the work reviewed above was on carotenogenesis. In our work we were interested in the effect of β-ionone on growth, sporulation, and glucose utilization as well as carotenogenesis. Growth was inhibited when 5 μl./flask of β-ionone (Fritzsche Bros.) was added at the time of inoculation. The β-ionone was dissolved in absolute alcohol and 10 μl. of the solution introduced along to the sides of the flasks by means of a 10 μl. pipet. Control experiments showed that 10 μl. of absolute ethanol was without effect on growth or carotenogenesis. The dry weights of mycelium produced by the control and β-ionone treated cultures after 6 days of incubation were 76.9 and 3.8 mg. respectively. The corresponding yields of carotene were 189 and 2μg. Lesser amounts of β-ionone were used in the other experiments.

Growth was decreased when 0.5 μl./flask of β-ionone was added 24 hours after inoculation, especially during the following 2 days of incubation. The pH values fell more slowly than in the controls and the initial rate of glucose utilization was depressed. More carotene was produced by the β-ionone-treated cultures than by the controls; the carotene content (7 days) of the treated cultures was 270 μg./culture, and of the control cultures 203 μg. When 0.5 μl. of β-ionone was added to 3-day-old cultures, it had no appreciable effect on growth. Slightly less carotene was produced than in the control experiments.

More detailed information from an experiment in which 1 μl. of β-ionone was used is presented (Figures 30 and 31). The β-ionone-treated cultures grew more slowly than the control cultures; this was particularly noticeable during the first days of incubation. The pH values of the β-ionone-treated culture filtrates fell much less rapidly than those of the controls. Glucose was used less rapidly by the β-ionone-treated cultures than by the controls. The β-ionone-treated cultures produced more carotene than the control cultures. These differences are magnified if the yield of carotene is calculated on the basis of μg. carotene/g. dry mycelium; the β-ionone-treated cultures contained 2,800 μg./g. while the control cultures contained only 295 μg./g. after 3 days of incubation; on the eighth day of incubation the β-ionone-treated cultures contained 4,300 μg./g. while the control cultures contained 3,150 μg./g.

When 1 μl. β-ionone was added to 4-day-old cultures some inhibition of growth and glucose utilization was noted; but it was much less than that found when β-ionone was added to 24-hour-old cultures. Carotenogenesis was inhibited; the carotene content of the control and 4-day-treated cultures were 168, 113 (5 days) and 235, 137 (8 days) μg./culture, respectively.

A number of experiments were made to determine whether cultures of *Phycomyces blakesleeanus* would use acetate after being treated with β-ionone. Three dosages of β-ionone were used: 10, 5, and 1 μl. per flask. The additions were made to 3-day-old cultures. The addition of 12.2 mg. of acidified potassium acetate was started on the fourth day. Four flasks from each set received 1 μc. of acetate-1-C^{14} on the fourth day of incubation. The total C.P.M. of the uninoculated control was 1.5×10^6. Twenty-four hours after adding labeled acetate the radioactivity (total C.P.M./flask) of the β-ionone-treated cultures were: 10 μl., 1.3×10^6; 5 μl., 9.6×10^4; 1 μl., 3.2×10^4; and control (no β-ionone), 4.4×10^4. These results indicate that 10 μl. of β-ionone inhibited almost completely the utilization of acetate, while 5 μl./flask or less had little or no effect on acetate utilization. In these experiments the addition of either 10 or

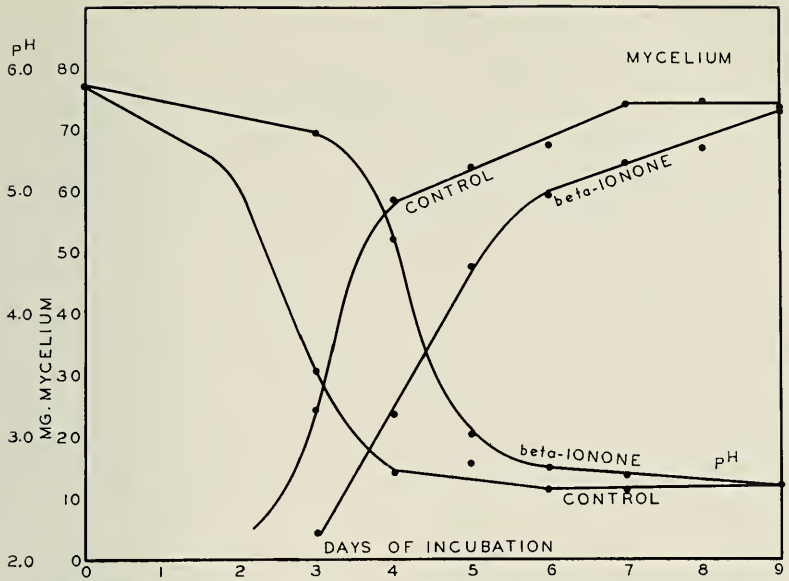

FIGURE 30. The effects of adding 1 μl. of β-ionone to 24-hour-old cultures on growth and change in pH values of the medium.

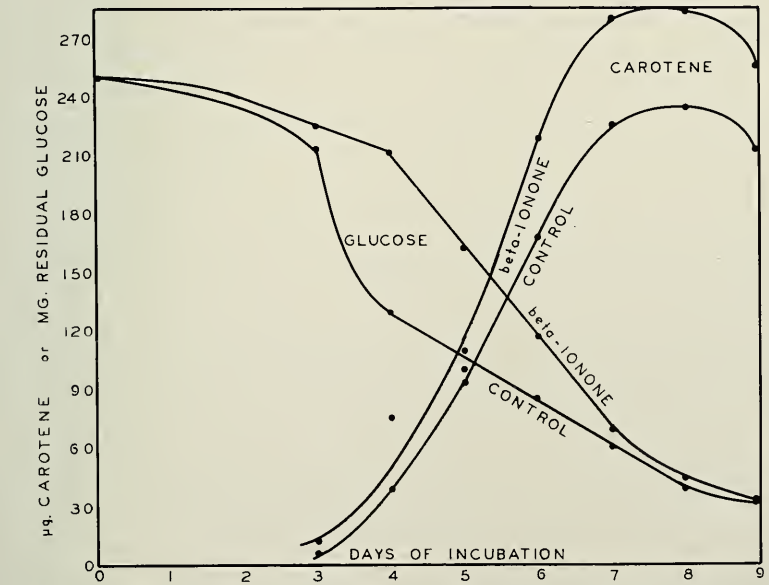

FIGURE 31. The effects of adding 1 μl. of β-ionone to 24-hour-old cultures on glucose utilization and production of carotene.

5 μl. of β-ionone effectively inhibited carotenogenesis. The addition of acidified potassium acetate one day after the addition of β-ionone did not overcome this inhibition. Indeed, the carotene content of the mycelium of cultures treated with 10 μl. of β-ionone and acidified potassium acetate decreased rapidly (5 μg./culture, 8 days) whereas the carotene content of cultures treated with 10 μl. of β-ionone alone remained constant (63 μg./culture, 8 days). The effects of 5 μl. of β-ionone were similar, but less severe.

β-Ionone and acidified potassium acetate decreased the rate at which glucose was utilized. These effects were additive.

The above experiments show that β-ionone may increase or decrease carotene synthesis depending upon the age of the cultures to which it is added and the concentration used. The effect of other variables must be considered also, especially the pH of the culture medium when β-ionone is added. Carotenogenesis was increased when the pH value was about 5.7 (24 hours) and decreased when the pH value was 2.7 (4 days).

Further experiments were made to determine whether the β-ionone effect was related to the pH of the culture medium. In these experiments the concentration of potassium acetate in the basal medium was increased from 0.72 to 1.94 g./l. This concentration of potassium acetate favors growth and sporulation, but is poor for carotenogenesis (Figures 2 to 5). The details are given in Table 3. One μl. of β-ionone was added after 24, 48, 72, and 96 hours. β-Ionone increased the carotene content when added at 24, 48, and 72 hours, and had little effect when added to 96-hour-old cultures. On the fifth day normal 2-inch long sporangiophores were present in the control cultures and absent in cultures treated with β-ionone at 24 hours. The sporangiophores in cultures treated with β-ionone at 48 hours were abundant but immature. The addition of β-ionone at 72 and 96 hours had little effect on the development of sporangiophores.

The action of β-ionone is rapid, most of the increased carotene content of the mycelium accumulates within 24 hours after β-ionone is added. Visual inspection of the cultures indicates that much of the stimulation occurs within 2 to 3 hours.

Whether the higher pH values of the modified basal medium were the factors responsible for prolonging the time during which β-ionone was an effective stimulator of carotenogenesis is not known. Relatively high yields (μg./culture) of carotene were obtained when the cultures were treated with β-ionone while the pH values were 4.5 or higher.

Table 3. The Effects of Adding 1 μl. of β-ionone at 24, 48, 72, and 96 Hours to Cultures of *Phycomyces blakesleeanus* Grown on a Modified Basal Medium Which Contained 1.94 g./l. of Potassium Acetate

Days	Mycelium mg.	pH	Residual Glucose mg.	Carotene μg./Culture	Carotene μg./g.
		Controls			
3	25.9	5.63	131	8	131
4	100.5	4.53	38	14	138
5	101.6	3.95	8	20	196
6	94.3	3.63	4	29	308
7	89.4	3.43	3	26	279
		24 *Hours*			
3	2.7	5.72	222	5	1,850
4	14.0	5.63	145	61	4,350
5	39.0	5.60	142	75	1,920
6	67.6	5.38	74	80	1,180
7	99.7	4.11	26	71	740
		48 *Hours*			
3	59.9	5.67	197	47	785
4	59.7	5.38	103	63	1,050
5	99.5	4.50	72	70	705
6	97.1	3.60	7	54	555
7	89.4	3.43	3	70	780
		72 *Hours*			
4	86.9	4.71	58	91	1,050
5	100.6	4.38	28	72	695
6	95.0	4.15	3	72	960
7	89.2	3.81	2	64	715
		96 *Hours*			
5	102.0	4.33	9	22	218
6	94.3	4.44	4	35	370
7	89.1	3.88	2	27	302

In experiments without β-ionone high yields of carotene were never obtained unless the pH of the culture medium was less than 3.

From the results obtained in these experiments, and others not reported, it was concluded that β-ionone is a toxic compound. The effects which follow treatment are dependent upon the age of the mycelium, the dosage, the pH of the culture medium, and no doubt to other factors. Mackinney *et al.* (1952) suggested that β-ionone stimulates carotenogenesis by blocking the pathways of biosynthesis which lead to the production of mycelium and sporangiophores, and leaving the pathways of carotene synthesis unaffected. This general conclusion appears to be valid, but it is far from a detailed answer to the action of β-ionone on *P. blakesleeanus* and other fungi.

β-Ionone appears to be a toxic compound with a wide spectrum of activity. The effects of β-ionone are dependent upon the dosage, the time of application, the composition of the basal medium, and no doubt

on other factors. In general, growth, asexual sporulation, and glucose utilization are inhibited; these effects are most noticeable when the mycelium is young and the pH of the medium is above 5.0. *Under certain conditions, carotenogenesi is markedly stimulated.*

MICRO ELEMENTS

Fungi require the following micro elements: iron, zinc, copper, manganese, molybdenum; some fungi require calcium; various other metallic elements have been reported to be required by a few species (Lilly and Barnett 1951). The usual criterion of essentiality is the reduction in growth which results when the concentration of a micro element is less than optimum. The study of micro element requirements is difficult and complex.

The use of growth as the sole criterion for determining the optimum concentration of a micro element to be used in a medium is valid for that purpose. However, the concentration optimum for growth may not be optimum for the production of a metabolic product such as riboflavin (Knüsel 1957), or for the production of various enzymes (Nason, *et al.* 1951). Micro element studies of fungi may be divided into two groups: (1) those in which a highly purified basal medium is used, and (2) less exacting studies in which various micro elements are added to a basal medium not specially purified. The response in the second type of experiments depends upon the micro element content of the basal medium. However, the method is useful in correcting micro element deficiencies of a particular basal medium.

Φdegard (1952) has reviewed the literature on the micro element nutrition of *Phycomyces blakesleeanus.* All of this work appears to have been done with non-purified media and indicates that *P. blakesleeanus* resembles other fungi in its micro element requirements.

By inadvertence, 1 × concentrations of micro elements were used in some recent experiments. The discovery of this error necessitated the repetition of a number of experiments. It also led to the recognition of the importance of micro element concentration in carotenogenesis and the routine use of the lower concentrations of micro elements. In the experiment to be reported the basal medium without added micro elements was used for the control medium. Iron, zinc, manganese, and calcium (2 ×) were added to one aliquot of the basal medium, and half that concentration to another aliquot. This procedure insured the same concentration of contaminating micro elements in all media. All cultures received 50 to 60 foot-candles of light. The temperature of incubation was 24° to 25° C.

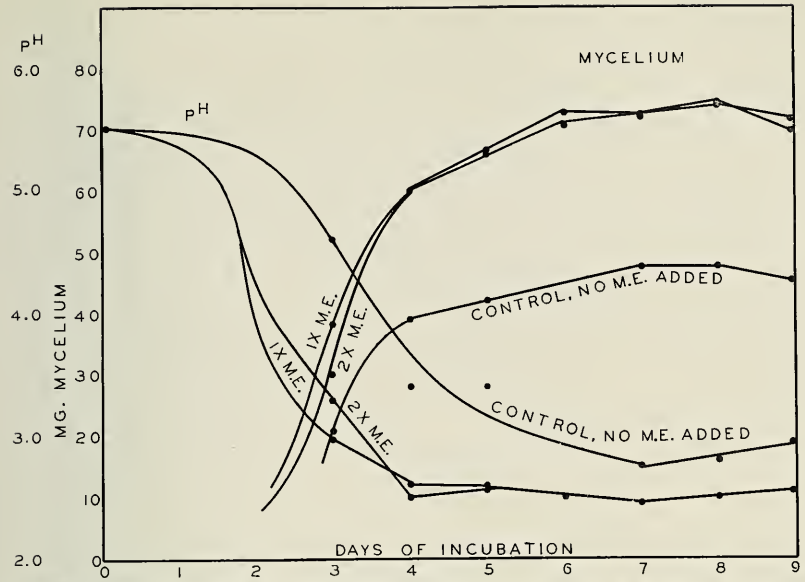

FIGURE 32. The effects of two concentrations of micro elements on growth and pH changes in the culture media.

The data for the production of mycelium and for the change in pH values are given in Figure 32. The weights of dry mycelium produced in the control medium without added micro elements was significantly less than that produced on either the 1 × or 2 × concentration of micro elements. So far as growth was concerned either concentration was equally satisfactory. The pH of the medium to which micro elements were not added did not decrease as much as in the other two media.

More glucose was utilized by the cultures grown on the media to which the two concentrations of micro elements were added than by cultures grown on media to which micro elements were not added; little or no effect of acetate addition on glucose utilization was noted in the control cultures.

The added micro elements had a greater effect on carotenogenesis than on growth. The yields of carotene are given in Figure 33. The addition of either 1 × or 2 × concentrations of the four micro elements resulted in a twofold increase in carotene production compared with the control cultures (no added micro elements). More carotene was synthesized in media containing 1 × concentrations of micro elements than when twice these concentrations were used. In view of these results, the use of 1 × concentrations were used in further work.

The above results suggested that a more intensive study of the micro element nutrition of *P. blakesleeanus* was needed. In order to investi-

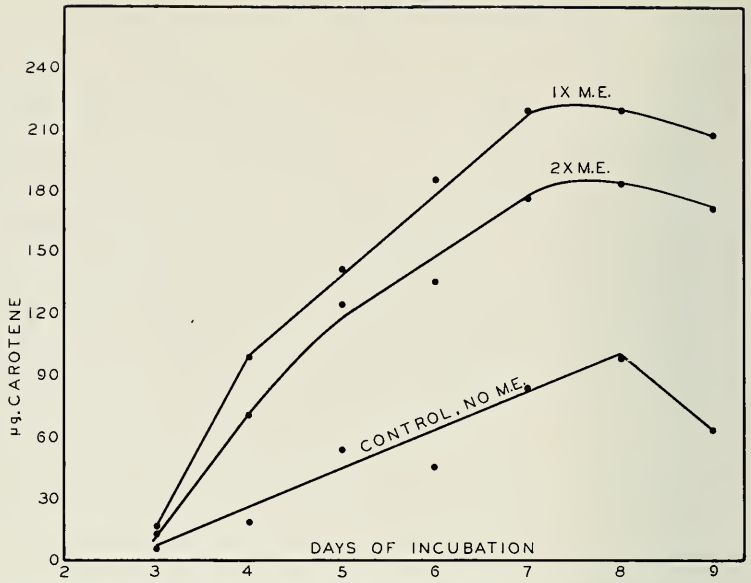

FIGURE 33. The effect of two concentrations of micro elements on the carotene content of mycelium of Phycomyces blakesleeanus.

gate this an experiment was designed using: iron and zinc, 0.1 mg./l. each; manganese, 0.05 mg./l.; and calcium, 10 mg./l., *i.e.*, 1 × concentrations. The data for growth and changes in pH values of the culture media are given in Table 4. A minute analysis of the data would show some differences among the control cultures and those which received the addition of zinc, iron, and both zinc and iron. However, about the same amount of growth was obtained under these four conditions. These results suggest the concentration of iron and zinc in the basal medium as contamination is sufficient for the production of about 50 mg. of mycelium per flask by the seventh or eighth day. When manganese was added in addition to zinc and iron, growth was increased. The addition of calcium to the other three micro elements resulted in increased growth.

The residual glucose concentrations after 8 days of incubation were: control, 124; zinc alone, 113; iron alone, 141; both zinc and iron, 117; zinc, iron, and manganese, 92; all four micro elements, 30 mg. Note that the addition of calcium which had only a small effect on growth markedly influenced the amount of glucose utilized.

The amounts of carotene synthesized by cultures grown on these combinations of micro elements differed greatly (Figure 34). The effect of adding iron alone to the basal medium was particularly striking, less carotene was synthesized (or accumulated) than when none of the four micro elements were added. This drastic reduction in the production of

Table 4. The Effects of Certain Micro Elements on the Growth and Change in pH Value of the Culture Medium of *Phycomyces blakesleeanus*

Days	No micro elements added		Zn		Fe		Zn and Fe		Zn, Fe, and Mn		Zn, Fe, Mn and Ca	
	Mycelium mg.	pH	Mycelium mg.	pH	Mycelium mg.	pH	Mycelium mg.	pH	Mycelium mg.	pH	Mycelium mg.	pH
3	29.5	5.80	25.7	4.42	25.2	5.27	31.0	3.54	35.1	3.10	43.6	2.80
4	41.8	3.99	49.5	2.88	36.5	5.19	54.7	2.86	58.1	2.68	60.8	2.59
5	39.6	3.30	43.4	2.85	32.6	4.68	51.8	2.75	61.3	2.63	65.4	2.50
6	40.6	3.28	49.1	2.78	41.9	3.25	54.9	2.65	62.3	2.40	70.1	2.38
7	53.6	2.90	52.7	2.70	44.7	3.30	51.9	2.60	64.9	2.50	74.6	2.40
8	55.0	2.93	54.1	2.88	53.3	3.05	53.8	2.58	66.9	2.50	77.0	2.45

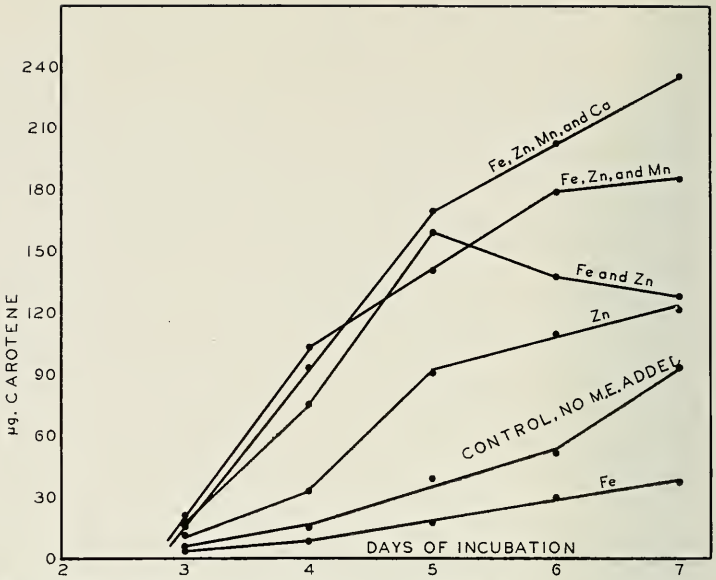

FIGURE 34. The effects of adding micro elements singly and in combination on the carotene content of the mycelium.

carotene may be ascribed to unbalanced micro element nutrition. The nature of "block" in carotene synthesis is unknown. The addition of zinc alone resulted in the production of more carotene than in the control cultures. When both iron and zinc were added still better yields of carotene resulted. Thus, in some way, zinc overcame the block in carotenogenesis that results when iron alone is added. As much carotene was synthesized by cultures receiving iron and zinc up to the fifth day of incubation as was produced by cultures receiving the four micro elements. This suggests that the basal medium contained sufficient concentrations of other micro elements to support rapid carotenogenesis up to the fifth day. After the fifth day of incubation, one or more of these essential micro elements were exhausted and the production of carotene ceased. The addition of manganese when both iron and zinc were supplied had little effect on carotenogenesis until after the fifth day of incubation. Likewise, the addition of calcium to media supplied with the other three micro elements had little effect on carotenogenesis until after the fifth day. The addition of calcium to the other three micro elements prolonged the period during which carotene was synthesized.

In the above experiments attention was called to the unknown micro element content of the basal medium. By treating the basal medium with dithizone (Donald, *et al.* 1952) it was possible to reduce the micro element contamination considerably. The next experiment reports a

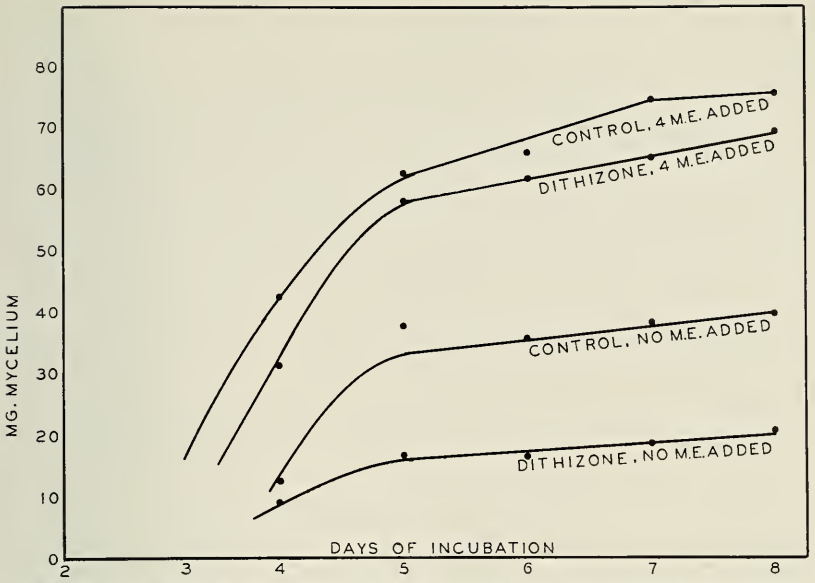

FIGURE 35. The effects of adding micro elements (1 ×) to untreated and dithizone-treated basal medium on the growth of Phycomyces blakesleeanus.

comparison of 4 levels of micro element nutrition. The following media were used: (1) the untreated basal medium without added micro elements, (2) untreated basal medium to which 1 × concentrations of the micro elements were added (3) dithizone-treated basal medium without the addition of micro elements, and (4) dithizone-treated basal medium to which 1 × concentrations of the micro elements were added.

The effects of these four treatments on growth are shown in Figure 35. Less mycelium was produced in the dithizone-treated control medium than in the untreated control medium. Treatment with dithizone resulted in a 47 per cent reduction in growth (8 days). Less mycelium was produced in the dithizone-treated medium to which micro elements were added than in the corresponding medium which had not been treated with dithizone. The most probable explanation appears to be that the untreated basal medium contained essential micro elements other than the four added in this experiment. It also is possible that the concentration of one or more of the added micro elements was reduced below optimum concentration.

The pH value of the dithizone-treated culture media was 5.33 on the eighth day; the corresponding pH value of the control medium not treated with dithizone was 3.33.

Glucose was utilized more completely (8 days) by cultures grown on untreated basal medium containing 1 × concentrations of the four micro

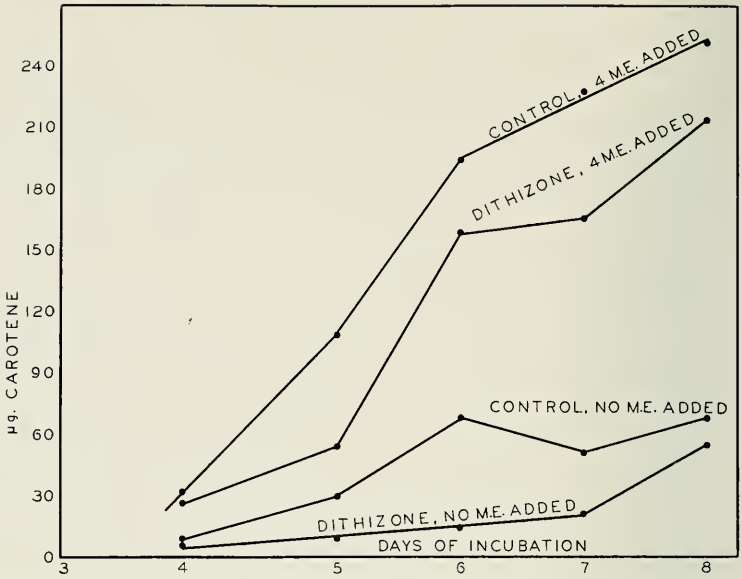

FIGURE 36. The effects of adding micro elements (1 ×) to untreated and dithizone-treated media on the production of carotene.

elements than by comparable cultures grown on dithizone-treated medium. The residual glucose after 8 days in the untreated basal medium was 32 mg.; in the dithizone-treated basal medium 62 mg.

The carotene yields for this experiment are given in Figure 36. These results emphasize the importance of micro element nutrition in the synthesis of carotene. The data suggest that some ions are removed by the dithizone treatment that stimulate carotenogenesis, or that the concentration of one or more of the ions routinely added was reduced below the optimum concentration. Preliminary experiments indicated that copper and molybdenum may be among the irons removed.

These experiments make it clear that carotenogenesis in *P. blakesleeanus* is influenced by micro element nutrition, and that much more work remains to be done on this subject.

Carotenogenesis in this isolate of Phycomyces blakesleeanus is influenced by the micro element content of the medium. Carotenogenesis is influenced by changes in the micro element composition that have little effect on growth. An excess of iron relative to the concentration of other micro elements depresses carotenogenesis, the addition of zinc overcomes the inhibition due to an excessive concentration of iron. Iron, zinc, manganese and calcium, and possibly copper and molybdenum, affect carotenogenesis.

General Discussion

The choice of a fungus for a biosynthetic process is of great importance. The fungus used should produce the desired compound in large amounts, be stable in culture, and be easy to culture. The ability of the fungus to utilize cheap and readily available C^{14}-substrates must be considered if the biosynthesis of C^{14}-labeled compounds is contemplated. When this work was begun in 1954, *Phycomyces blakesleeanus* was chosen since more information was available concerning carotenogenesis by this species than by any other. It was known that *P. blakesleeanus* would utilize acetate as a sole source of carbon, and that acetate-carbon was incorporated into β-carotene, the chief carotenoid synthesized by this fungus.

In the past few years, the production of carotene by a number of fungi has been studied in detail. *Blakeslea trispora* and *Choanephora cucurbitarum,* under certain conditions, produce more carotene than *P. blakesleeanus.* However, the utilization of acetate by these species has not been thoroughly investigated. All of the available information indicates that nutritional and environmental factors must be closely controlled if high yields of carotene are to be obtained with these species. It should be emphasized that the optimum conditions for carotenogenesis for each fungus must be determined, and that these conditions differ.

The synthesis of carotene appears to be a normal metabolic activity of *P. blakesleeanus* since carotene was produced under all of the experimental conditions tried. The role of carotene in the life processes of the fungus is not known. The amount of carotene produced when the cultures made normal growth and produced long sporangiophores was low (200 to 400 μg./g.); when growth was restricted and the production of sporangiophores was inhibited much more carotene was synthesized. This suggests that the amount of carotene necessary for the normal physiological activity of the fungus may be small, if at all necessary, compared with the potential ability of the fungus to synthesize this compound. It would be expected by analogy that physiologically active carotene would be associated with one or more proteins; under conditions suboptimal for growth and sporulation the excess carotene accumulates in oil droplets. This accumulation is not a serious argument against a physiological role of carotene. Many physiologically active metabolites are known to be synthesized in excess of requirement, particularly under conditions unfavorable for normal development. Water soluble metabolites such as riboflavin may be excreted in such quantities that it crystallizes from the medium (Knüsel 1957). Also, the cells of *Ustilago sphaerogena* may become pink due to the accumulation of cytochrome C when

the fungus is cultured under certain conditions (Grim and Allen 1954).

The production of high yields of carotene are associated with the use of conditions which are unfavorable for the rapid production of mycelium, utilization of glucose, and asexual sporulation. These conditions appear to block partially some normal metabolic processes and leave open the pathways which lead to the synthesis and accumulation of carotene. The biochemical pathways which result in the maximum production of mycelium and sporulation are inhibited by low external pH values of the medium and by the addition of certain concentrations of β-ionone. Since β-ionone may reduce growth and increase the accumulation of carotene when the pH values of the nutrient solution are high, it may be deduced that the effects of low external pH values and β-ionone are indirect. Not all substances that inhibit growth stimulate carotenogenesis. Diphenylamine in certain concentrations has relatively little effect on growth but largely inhibits the synthesis of β-carotene. Fluorides inhibit *both* growth and carotenogenesis. These results suggest that β-ionone, diphenylamine, fluoride, and low pH values produce their effects by inhibiting different enzyme systems.

From the biochemical standpoint, acetate is one of the most interesting and versatile of metabolites. Acetate has been known to enhance the growth of *P. blakesleeanus* on a glucose-ammonium nitrogen medium since 1940 (Leonian and Lilly). Within certain limits of concentration, this effect cannot be ascribed to the buffer action of acetate since the pH values are actually lower when acetate is added to a glucose-ammonium sulfate medium (Figure 3) than when acetate is omitted from the medium. Furthermore, acetate is utilized by *P. blakesleeanus* so that this compound plays an active rather than a passive role. Glucose utilization is increased. Why and how this effect is produced is not known. Other examples of the favorable effect of mixed carbon sources could be cited, but a satisfactory explanation for this common phenomenon is lacking. While the effects of low concentrations of acetate on growth can be simulated by partially neutralizing the medium, carotenogenesis is is decreased.

The analytical data for the carotene content of the mycelium are a measure of the total carotene present at the time of analysis. It is tempting to equate these values with the amount of carotene synthesized. While it is known that the carotene content of cultures decreases with age, it is not known whether carotene is being actively metabolized in younger cultures. So long as the carotene content of the mycelium is increasing, it can be said that the rate of synthesis exceeds the rate of destruction or utilization. It is possible that low accumulation of carotene under certain

conditions does not reflect accurately the amount of carotene actually synthesized. An answer to this problem would be of interest in any attempt to evaluate the possible roles of carotenoids in fungi.

Summary, Part I

Nutritional and environmental factors influence markedly the amount of carotene accumulated by cultures of *Phycomyces blakesleeanus*. Some carotene was produced under all of the conditions tried.

It was confirmed that *P. blakesleeanus* would utilize acetate as a sole source of carbon for growth and carotenogenesis. Growth was limited, and the amount of carotene produced was small when the acetate medium was used. Growth was limited when a glucose-ammonium sulfate medium was used; the yield of carotene per unit weight of mycelium was high although the amount produced per culture was unsatisfactory.

The addition of potassium acetate to the glucose-ammonium sulfate medium improved the basal medium. The effects depended upon the concentration of acetate added; the dry weight of mycelium increased as the concentration of acetate increased from 0 to 0.02 *M*. Normal asexual reproduction occurred when the acetate concentration was between 0.02 and 0.04 *M*; asexual reproduction was greatly restricted when the concentration of acetate was 0.01 *M* or less. The pH values of culture media containing 0.01 *M*, or less, of potassium acetate were lower during incubation than those of the control medium (no added acetate). The rate of glucose utilization and the amount of glucose used increased as the concentration of acetate increased from 0 to 0.02 *M*. The yields of carotene per culture increased as the concentration of acetate increased from 0 to 0.01 *M*; greater concentrations resulted in decreased yields of carotene, and very little carotene accumulated when the acetate concentration was 0.02 *M* or greater. A concentration of 0.0075 *M* potassium acetate was used thereafter in the basal medium.

Phycomyces blakesleeanus is not a suitable fungus for the production of carotene in shake cultures. Agitation sufficiently severe to disturb the mats results in decreased growth and carotene production. Very low yields of carotene were obtained in aerated liquid culture. It is thought that agitation reduces the availability of oxygen to the mycelium. Surface mycelium appears to contain more carotene than submerged mycelium.

The addition of yeast extract (1 g./l.) to the basal medium results in increased growth and a more rapid initial rate of carotenogenesis. The daily addition of glucose (7.5 to 15 mg./culture) prolongs the period during which carotene is synthesized and to prevent or delay the

loss of carotene which occurs in cultures following the exhaustion of glucose.

The pH levels that prevail during incubation are important for the production of carotene. When the culture medium of 4-day-old cultures was partially neutralized with sodium carbonate, growth was increased; but the synthesis of carotene was greatly decreased. The decrease was proportional to the amount of sodium carbonate added. Also, various quantities of potassium acetate were added to 4-day-old cultures to increase the pH values of the culture medium. Low concentrations of potassium acetate (2 to 6 mg./culture) resulted in a slight increase in carotene production; higher concentrations decreased the yield of carotene. When sufficient sodium carbonate or potassium acetate was added to increase the pH levels above 3.0, both compounds had about the same effect.

The initial pH values of the basal medium affected growth and carotenogenesis. Satisfactory yields of carotene were obtained in the pH range of 5.5 to 6.0; however, initial pH values between 5.5 and 5.75 appeared to be best.

Cultures of *P. blakesleeanus,* from 4 to 7 days old, utilized as much as 7.5 mg. of acetic acid daily. Acidified potassium acetate was used instead of acetic acid since it was easier to prepare. If the acidified potassium acetate was carefully mixed with the culture medium as much or more carotene was produced as that obtained from the control cultures. The daily addition of acidified potassium acetate reduced the amount of glucose utilized. This sparing effect of added acidified potassium acetate on glucose utilization was found in all experiments. These effects are very different from those found when this amount of potassium acetate (not acidified) was added to 4-day-old cultures.

This isolate of *P. blakesleeanus* (104) used in this work produced more carotene than the three other isolates tested. There was a slight increase in carotene production when plus and minus isolates were grown together, but this occurred only under certain environmental conditions.

Light and temperature are important environmental factors which must be carefully controlled. Cultures grown in light and darkness at 25° C. produced about the same amount of mycelium; this was not unexpected since light affects vegetative growth of only a few fungi. More carotene was produced when the cultures were illuminated (50 to 60 foot-candles) continuously than under any other conditions tried. More carotene was produced at 25° to 26° C. than at any other temperature investigated. Carotene production was reduced about 50 per cent when the cultures were incubated at 20° C. About the same amount of my-

celium was produced at all temperatures between 20° and 28° C. More asexual sporulation occurred at 20° C. than at higher temperatures.

The reports that β-ionone stimulate carotenogenesis by *P. blakesleeanus* were confirmed. The effects of β-ionone depended upon the amount used, the time of addition, and the composition of the medium. The addition of 1 μl. of β-ionone to 1-day-old cultures growing on the basal medium resulted in increased production of carotene, decreased growth, and reduced utilization of glucose. Addition of 1 μl. of β-ionone to 4-day-old cultures was largely without effect. The addition of β-ionone to cultures growing on a modified basal medium containing 0.02 *M* acetate also increased carotene production. This increased production of carotene when the modified basal medium was used occurred when the pH values were high (ca 5). β-Ionone appears to simulate the effects of low pH values in increasing the production of carotene. While the mode of action of β-ionone is not known, it appears to restrict the biosynthetical pathways involved in the production of mycelium and leave unaffected the pathways of carotene synthesis.

Carotenogenesis by *P. blakesleeanus* is affected more by the concentration of micro elements in the medium than is growth. A proper balance among the concentrations of micro elements is necessary. Excessive concentrations of iron when low concentrations of the other micro elements were present severely depressed the yield of carotene.

When the basal medium was used and the cultures incubated at 25° to 26° C. under continuous illumination (50 to 60 foot-candles) for 6 to 8 days, the yield of carotene was approximately 3,000 μg./g. By adding 1 μl. of β-ionone to 24-hour cultures the yield was increased to 4,000 μg./g. These yields are as high, or higher, than those reported by other investigators for other isolates of *P. blakesleeanus*.

PART II: The Production of Radioactive beta-Carotene Using *Phycomyces blakesleeanus*

The experiments reported in Part I of this Bulletin had for their purpose the establishing of suitable conditions for the production of carotene by *Phycomyces blakesleeanus*. This work served as the basis for the following experiments.

LITERATURE REVIEW

Apparently, only two species of fungi (*P blakesleeanus* and *Mucor hiemalis)* have been used for the biosynthesis of radioactive β-carotene. These investigations were primarily designed to produce radioactive β-carotene for chemical studies and to trace the pathways of biosynthesis.

Grob *et al.* (1951) were the first to use a fungus for the production of radioactive β-carotene. *P. blakesleeanus* was cultured on a lactate-acetate medium, a yield of 35 mg. dry mycelium was obtained from 25 ml. of medium. The yield of carotene was 645 μg./g. dry mycelium. Acetate-1-C^{14} and acetate-2-C^{14} were used in separate experiments. No data were given for the amount of acetate utilized under these conditions. Grob *et al.* (1952) reported *P. blakesleeanus* to utilize the methyl and carboxyl carbons of acetate equally for the synthesis of radioactive β-carotene. Under these conditions, it was calculated that 69.5 per cent of the carbon in β-carotene was derived from acetate, 33 per cent from the methyl carbon and 36.5 per cent from the carboxyl carbon. The remainder of the carbon was assumed to be derived from carbon dioxide. The assumption has apparently not been verified. The lipids in the mycelium were labeled with C^{14}, but no data were reported concerning the relative amounts of C^{14} found in β-carotene, fats, and sterols.

Glover *et al.* (1951) found 4-day-old mats of *P. blakesleeanus* when transferred to a glucose-sodium acetate solution to synthesize radioactive β-carotene. Acetate-2-C^{14} was used in these experiments. In other experiments where mats were transferred to a medium containing acetate as the sole source of carbon, it was found that acetate was dissimulated; C^{14} was found in the lipids, proteins, carbohydrates and carbon dioxide. Only a little radioactive β-carotene was synthesized under these conditions.

Mevalonic acid (β-hydroxy-β-methyl-δ-valerolactone), a recently discovered acetate replacing factor for certain *Lactobacilli,* has been shown to be the precursor of β-carotene in *Mucor hiemalis* by Grob (1957) and in *P. blakesleeanus* by Braithwaite and Goodwin (1957). Both investigators used mevalonic acid-2-C^{14}. Braithwaite and Goodwin (1957) report that not all of the mevalonic acid added to the medium was utilized; they ascribed this to a permeability effect. They reported that mevalonic acid was incorporated into the β-carotene molecule about ten times as efficiently as acetate-2-C^{14} under the same experimental conditions.

C^{14}-Labeled sugars have been used to produce radioactive β-carotene using *P. blakesleeanus.* Chichester *et al.* (1954) added fructose-u-C^{14} to a glucose-yeast autolysate medium. *P. blakesleeanus* produced radioactive β-carotene under these conditions. Chichester *et al.* (1956) investigated the biosynthesis of radioactive β-carotene on a glucose-glycine medium. Glucose-u-C^{14}, glycine-1-C^{14}, and glycine-2-C^{14} were used as sources of C^{14} in separate experiments. It was concluded that *P. blakesleeanus* used 38 carbon atoms from glucose and 2 carbon atoms from glycine for the biosynthesis of β-carotene (C_{40}) when this medium was

used. Glycine-carbon was not incorporated as a two-carbon unit since 2-C was incorporated with twice the efficiency of 1-C. Leucine labeled with C^{14} in various positions was used by Chichester *et al.* (1955) and Yokoyama *et al.* (1957) for the biosynthesis of β-carotene by *P. blakesleeanus*. The unequal incorporation of C^{14} from the C^{14}-isomers of leucine demonstrated that leucine did not furnish directly a C_5 precursor of β-carotene.

Mucor hiemalis was used by Grob and Bütler (1954, 1954-a) for the biosynthesis of radioactive β-carotene. The basal medium used contained acetate as the sole source of carbon (Schopfer and Grob 1952). The methyl and carboxyl carbons of acetate were shown to enter the β-carotene molecule equally. The yield of β-carotene was about 25 μg./25 ml. The evidence from degradation experiments showed that the methyl and carboxyl carbons of acetate occupied different positions in the β-carotene molecule. This evidence makes it probable that *M. hiemalis* incorporates acetate as a two-carbon moiety into β-carotene. This does not necessarily mean that acetate was directly incorporated into the β-carotene molecule.

Apparently, no intensive degradation studies of radioactive β-carotene produced by *P. blakesleeanus* from acetate have been published. In the absence of specific information it is natural to assume that the pathways of carotene biosynthesis by *M. hiemalis* and *P. blakesleeanus* are the same. Whether this assumption is correct or not must await a direct examination of this hypothesis.

PRELIMINARY EXPERIMENTS

A number of experiments for the production of radioactive β-carotene were made before the importance of light and temperature were fully realized. These experiments demonstrated that the time at which C^{14}-labeled acetate or C^{14}-labeled glucose were added to the cultures was important. When C^{14}-labeled acetate was added at the time of inoculation the β-carotene isolated had a specific activity of about 3,000 C.P.M./mg. When the C^{14}-labeled acetate was added on the fourth day of incubation the specific activity of the β-carotene was much higher. Glucose-u-C^{14} and formate-1-C^{14} were used in a few experiments. The radioactivity of the β-carotene isolated in these experiments was low.

The effects of adding labeled acetate at the time of inoculation and to 4-day-old cultures on the specific activity of β-carotene are readily explicable when the data for the rate and amount of C^{14}-labeled acetate utilized are viewed in conjunction with a time curve for growth and the synthesis of carotene. (Figure 37). The synthesis of carotene is extremely slow during the first three days of incubation, when essentially all of the C^{14}-labeled acetate had been utilized. By the time the culture began to

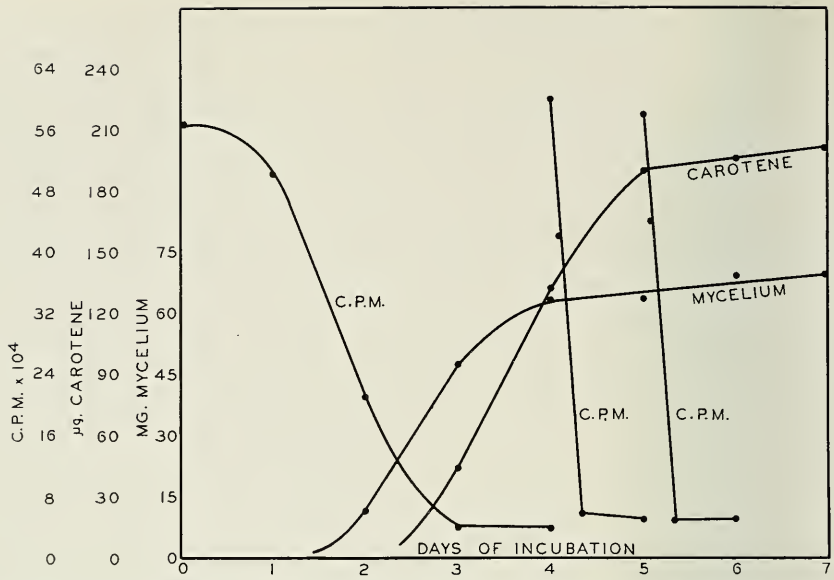

FIGURE 37. Rates of utilization of acetate as indicated by the residual radioactivity of the medium during growth and carotenogenesis. Acetate-1-C^{14} was added at 0, 4, and 5 days.

synthesize appreciable amounts of carotene only a trace of C^{14}-labeled acetate remained in the medium. When C^{14}-labeled acetate was added on the fourth day of incubation, mycelial growth was essentially complete but carotenogenesis was rapid. When C^{14}-labeled acetate was added on the fourth and fifth days of incubation, most of it was used within 8 hours. The residual radioactivity after 24 hours of incubation with C^{14}-labeled acetate was probably due to the presence of labeled compounds other than acetate since rapid utilization of acetate occurred when it was added on the fifth day of incubation. The data for the utilization of C^{14}-labeled acetate on the fourth and fifth day of incubation are from the same cultures (Figure 37).

In order to obtain radioactive β-carotene with a high specific activity it is essential to add labeled acetate after the growth phase is largely over. C^{14} from formate is not incorporated appreciably into β-carotene.

ISOLATION OF β-CAROTENE

The method (Lilly *et al.* 1958) of obtaining pure β-carotene from the mycelium of *P. blakesleeanus* is described below. Twenty-eight hours after the addition of C^{14}-labeled acetate to the medium, the mycelium was collected on a fluted filter, pressed damp dry, and placed in absolute methanol. This treatment dehydrated the mycelium so that the carotene

could be extracted with petroleum ether. After dehydration, the mycelium was thoroughly extracted with redistilled petroleum ether (b.p. 35° to 37° C.). The mycelium and petroleum ether were cooled to −20° C. before extraction in a Waring blender. Successive additions of cold petroleum ether were made until the extracts were colorless. At this point, the total volume of extract was measured and the carotene content and radioactivity were determined. This method of extracting carotene was as efficient as grinding the mycelium with diethyl ether as was shown by the close correspondence between the carotene values of the control cultures (extracted with redistilled diethyl ether) and the carotene content of the petroleum ether extract.

The next step was saponification. The saponification procedure, as well as the other steps in the purification procedure, were carried out under dim red light. The petroleum ether extract was concentrated under reduced pressure to about 30 ml. in a Rinco evaporator. The concentrated extract was saponified by refluxing with 2 *N* potassium hydroxide in ethanol for 90 minutes.

The saponification mixture was cooled and diluted with an equal volume of distilled water, and the nonsaponifiable compounds extracted with petroleum ether. The petroleum ether solution was extracted with aqueous N/2 potassium hydroxide to remove traces of fatty acids, and then washed with distilled water until the washings were neutral to phenophthalein. The carotene content and the radioactivity were usually measured at this time.

The petroleum ether solution of the nonsaponifiable compounds was dried with anhydrous sodium sulfate. The solution was filtered and treated with tricalcium phosphate (Mitchell *et al.* 1953), and they passed through a column of bone meal (Glover *et al.* 1958). The petroleum ether was removed by vacuum distillation and the solid residue washed 5 times with 5 to 10 ml. portions of cold methanol which contained 1 per cent of petroleum ether. The methanol insoluble material was dissolved in petroleum ether and concentrated under vacuum to a volume of 30 ml. β-Carotene usually began to crystallize during the evaporation of the petroleum ether extract. The petroleum ether solution was kept in darkness at −20° C. for 24 to 48 hours. The crystals of β-carotene were collected on a sintered glass funnel and washed 5 times with 5 to 10 ml. portions of cold methanol. The crystals were dried, weighed, and then redissolved in petroleum ether. The optical density and radioactivity were determined on suitable aliquots. The solution of β-carotene was taken to dryness under nitrogen. The β-carotene was stored under nitrogen in the dark at −20° C.

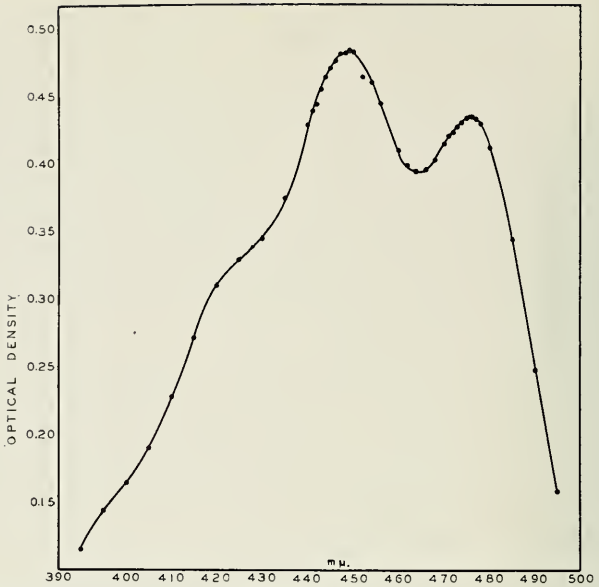

FIGURE 38. The absorption spectrum of a sample of β-carotene isolated and and purified by the methods described in the text.

The criterion of purity used in this work was the E (1%, 1 cm.) value. This value was calculated from the optical density (450 mμ.) of the final petroleum ether solution and w/v concentration of this solution by the use of the following formula:

$$E\ (1\%,\ 1\ \text{cm.}) = \frac{\text{optical density}}{\text{conc.}\ (1\%) \times \text{length of cell in cm.}}$$

Various E (1%, 1 cm.) values for β-carotene have been reported. Booth (1957) suggests that 2,550 is a representative value.

The spectra of a number of samples of β-carotene prepared by the above method were obtained with a Beckman model DU spectophotometer. The absorption curves obtained corresponded closely with curves given for β-carotene in the literature (Booth 1957; Goodwin 1954). An absorption curve for β-carotene purified by the above procedure is given in Figure 38.

The data for six consecutive experiments in which radioactive β-carotene was prepared are presented in Table 5. The experiments, are listed in the order that they were done. In recent experiments the final yield of pure β-carotene has been about 50 per cent of that present in the first petroleum ether extract. This improvement in yield is to be

Table 5. Loss of Carotene During Purification in 6 Consecutive Experiments. Carotene Values in mg.

No.	Petroleum Ether Extract	After Saponification	After Treatment With Calcium Triphosphate and Bone Meal	After Cold Methanol Wash	Crystalline β-Carotene	Per Cent Loss
1	17.1	—	12.2	10.4	5.0	70.8
2	13.8	10.1	—	4.8	4.6	67.0
3	16.1	15.2	—	8.4	6.8	57.2
4	15.5	13.0	13.7	7.9	5.6	63.9
5	17.6	—	17.8	13.6	9.0	49.0
6	19.9	19.9	18.7	15.0	11.5	42.2

attributed to the more extensive use of dim red light and protection of carotene from air.

The E (1%, 1 cm.) values were 2,563, 2,009, 2,559, 2,522, 2,495, and 2,749. Difficulty was experienced with the spectrophotometer or micro balance in the two experiments where E (1%, 1 cm.) values of 2,009 and 2,749 were found. If these experiments are excluded the average E (1%, 1 cm.) value of β-carotene was 2,535.

It may be noted that the carotenes are labile substances which are difficult to purify. The losses during purification by the method used in this work are higher than desirable, but not unexpected, in view of the ease with which β-carotene is destroyed by light and oxygen. This method resulted in the isolation of β-carotene having better E (1%, 1 cm.) values than when purification was carried out by column chromatography.

By the method described it was possible to isolate crystalline β-carotene of high purity.

UTILIZATION OF C^{14}-LABELED ACETATE

An evaluation will be made in this section of the efficiency with which *Phycomyces blakesleeanus* incorporated C^{14}-labeled acetate into β-carotene, and other lipids. These considerations are important from the standpoint of improving the efficiency of incorporation and also from the standpoints of cost and time. On the average, about two weeks are required for the production of a sample of radioactive β-carotene. One week is required to make the medium, grow the cultures, and harvest the radioactive mycelium. Another week is required for the purification and isolation of the radioactive β-carotene. The cost of obtaining radioactive β-carotene may be considered under two headings. The *fixed* cost is that required for growing the cultures and isolating the β-carotene. The *variable* cost is a function of the amount of C^{14}-labeled acetate used. Since the activity of the β-carotene, within limits, depends upon the amount of C^{14}-labeled acetate used, it is important to use conditions in which the per cent of incorporation of C^{14} into β-carotene is high. Much remains to be done on this phase of the investigation.

The experimental conditions used were alike in that the temperature was maintained between 24.5° and 26° C. during incubation. The cultures were illuminated 24 hours daily (50 to 60 foot-candles). The daily addition of 12.2 mg. of acidified potassium acetate was started one day before C^{14}-labeled acetate was added. The cultures were carefully tilted after the addition of acetate. The radioactive mycelium was harvested 28 hours after the last addition of acetate.

The variables included adding different amounts of C^{14}-labeled

acetate and the time of incubation at which it was added. The time of harvest in most experiments was six days, but other times were used in some experiments. This information is collected in Table 6.

Table 6. The Amount and Kind of Radioactive Acetate Used, the Time of Addition, and the Number of Flasks Used in 6 Experiments for the Production of Radioactive β-Carotene

No.	Kind and Amount of C^{14}-labeled Acetate Used	Day of Addition of C^{14}-labeled Acetate To Cultures	Number of Flasks Used
1	2 mc., acetate-1-C^{14}	4	100
2	0.1 mc., acetate-2-C^{14}	5	110
3	2 mc., acetate-1-C^{14}	5	79
4*	60 μc., acetate-1-C^{14} 60 μc., acetate-2-C^{14}	5	78
5	0.2 mc., acetate-1-C^{14}	5	92
6	3 mc., acetate-1-C^{14} (1 mc. on each of 3 days)	5, 6, 7	92

*In experiment 4 both acetate-1-and-2-C^{14} were used.

The concentration of micro elements was 2 × in experiments 1 and 2, and 1 × in others.

The essential data relating to the production of mycelium, the pH values of the culture medium, the amount of residual glucose, and the yield of carotene on the day of harvest are presented in Table 7.

Table 7. The Data for the Production of Mycelium, pH Values of the Culture Filtrates, Residual Glucose, and the Yield of Carotene in 6 Consecutive Experiments for the Production of Radioactive β-Carotene. All Data are on a Flask Basis Except Total Carotene

No.	Day of Harvest	Mycelium mg.	pH	Residual Glucose mg.	Carotene μg.	Carotene μg./g.	Carotene (total) mg.
1	5	72.6	2.54	—	171	2,370	17.1
2	6	65.1	2.85	102	125	1,925	13.8
3	6	57.8	2.44	51	192	3,400	15.2
4	6	64.7	2.50	90	197	3,010	15.5
5	6	66.8	2.50	73	192	2,880	17.6
6	8	75.8	2.55	63	216	2,870	20.0

The value of radioactive β-carotene in many chemical and in most biological applications depends upon a sufficiently high specific activity (C.P.M./mg.). From this standpoint, it is convenient to evaluate the data upon the basis of yields and specific activities of the β-carotene produced or isolated. The β-carotene isolated consists of a mixture of non-radioactive β-carotene synthesized *before* labeled acetate was added,

and radioactive β-carotene synthesized *during* the terminal 28 hours. The over-all efficiency is calculated upon the basis of the total activity (C.P.M.) used and the total activity of the mixture of radioactive and non-radioactive β-carotenes produced or isolated.

DISTRIBUTION OF C^{14} FROM LABELED ACETATE

The following measurements of radioactivity (as total C.P.M.) were made. Total C.P.M. added to the cultures and the amount of radioactivity remaining in the culture filtrate at the time of harvest. From these data the total C.P.M. used by the cultures in each experiment was calculated. The radioactivity of the petroleum ether extract of the radioactive mycelium was determined. The C.P.M. of β-carotene in the petroleum ether extract was calculated by multiplying the mg. of β-carotene by the specific activity (C.P.M./mg.) of the pure β-carotene isolated. It is felt that this procedure is justified since it avoids the losses in carotene suffered during purification and our present interest is in the utilization of acetate by fungus. These data are collected in Table 8 and are expressed in terms of per cent in Table 9.

The data in Table 9 shows that the per cent of added radioactive acetate utilized varied from experiment to experiment; in 5 of the 6 experiments from 93.2 to 97.9 per cent was utilized. These values may be low since the radioactivity of the culture filtrate has never been observed to fall to zero. It is considered probable that the residual radioactivity is due to C^{14}-labeled metabolic products secreted into the medium. Most of the added radioactive acetate was used as a source of energy and lost as carbon dioxide, or incorporated into non-lipid compounds. At present, we have no data on the amount of C^{14} converted into carbon dioxide.

The per cent of C^{14} incorporated into petroleum ether soluble compounds varied from 8.5 to 28.6 per cent (Table 9). The compounds extracted from the mycelium included fats, sterols, and carotenoids. There appeared to be no correlation between the radioactivity of the petroleum ether extract and the radioactivity of β-carotene. These results suggest that there are many factors, at present unknown, which influence the amount of C^{14} incorporated into β-carotene.

The radioactivity of the nonsaponifiable fraction was determined in 4 of the 6 experiments. This information is collected in Table 10. From these data it will be seen that about 80 to 85 per cent of the C^{14} incorporated into the petroleum ether soluble compounds entered the saponifiable fraction. These data suggest that of every 100 C^{14} atoms incorporated into the petroleum ether soluble compounds, from 80 to 85 were incorporated into fats or fatty acids.

Table 8. The Utilization of Radioactive Acetate by *Phycomyces blakesleeanus*. Radioactivity is Given as Total C.P.M

No.	C.P.M. Added to Cultures	C.P.M. Remaining in the Filtrate at Time of Harvest	C.P.M. In Petroleum Ether Extract	C.P.M. in β-Carotene
1	$1{,}418 \times 10^6$	317×10^5	370×10^6	173×10^5
2	55×10^6	729×10^4	101×10^5	54×10^4
3	$1{,}106 \times 10^6$	227×10^5	916×10^5	731×10^4
4	88×10^6	596×10^4	135×10^4	119×10^4
5	$1{,}302 \times 10^5$	879×10^4	$1{,}656 \times 10^4$	234×10^4
6	$1{,}725 \times 10^6$	579×10^5	$1{,}756 \times 10^5$	124×10^5

Table 9. Per Cent of Added Radioactive Acetate Utilized and the Per Cent of C^{14} Incorporated into Petroleum Ether Soluble Compounds Including β-Carotene

No.	Per Cent Utilized	Per Cent Incorporated Into Petroleum Ether Soluble Compounds	Per Cent Incorporated Into β-Carotene	Per Cent In Non-lipid Compounds in Mycelium and as CO^2 (calculated)
1	97.8	26.7	1.25	73.3
2	86.7	21.2	1.13	78.8
3	97.9	8.5	0.68	91.5
4	93.2	16.4	1.45	85.6
5	93.2	13.6	1.93	86.4
6	96.6	10.5	0.74	89.5

The distribution of C^{14} in the nonsaponifiable fraction can be calculated in 4 experiments for which data are available. These data are given in Table 11.

Since the yield of carotene is low, it is to be expected that the efficiency of incorporation of C^{14} into β-carotene will be low. The yield from acetate in the various experiments is calculated in terms of the number of C^{14} carbon atoms required for the incorporation of one C^{14} atom into the β-carotene molecule (Table 12).

Further study is needed to define the conditions under which acetate carbon is incorporated to the greatest extent into β-carotene.

Table 10. Distribution of Radioactivity in the Petroleum Ether Extract Between the Saponifiable and Nonsaponifiable Fractions. Activities are Given as C.P.M. and as Per Cent

No.	C.P.M. in Petroleum Ether Extract	Saponifiable Fraction		Nonsaponifiable Fraction	
		C.P.M.	Per Cent	C.P.M.	Per Cent
2	101×10^5	868×10^4	85.9	142×10^4	14.1
3	916×10^5	738×10^5	80.6	178×10^5	19.4
4	135×10^5	$1{,}167 \times 10^4$	84.4	183×10^4	13.6
6	$1{,}756 \times 10^5$	$1{,}395 \times 10^5$	79.4	361×10^5	20.6

Table 11. Distribution of Radioactivity in the Nonsaponifiable Fraction. Activities are Given as Total and as Per Cent

No.	In Nonsaponifiable Fraction	Sterols, Other Compounds		β-Carotene	
		Total	Per Cent	Total	Per Cent
2	142×10^4	102×10^4	77.9	40×10^4	28.1
3	178×10^5	$1{,}095 \times 10^4$	61.6	685×10^4	38.4
4	183×10^4	83×10^4	45.4	100×10^4	54.6
6	361×10^5	237×10^5	65.7	124×10^5	34.3

Table 12. Incorporation of C^{14} from Labeled Acetate Into β-Carotene. The Calculations are Based on the Total C.P.M. of the β-Carotene Produced and the Total C.P.M. Utilized by the Cultures

No.	Per Cent of Acetate Carbon Incorporated Into β-Carotene	Ratio of C^{14} Atoms Incorporated Into β-Carotene With Respect To Total Number Utilized
1	1.25	1/80
2	1.13	1/89
3	0.68	1/147
4	1.45	1/69
5	1.93	1/52
6	0.74	1/135

In the 6 experiments reported above the per cent of C^{14} from acetate incorporated into β-carotene ranged from 1.93 to 0.74 per cent (Table 12).

Inasmuch as different amounts of C^{14}-labeled acetate were used in the various experiments the specific activity of the radioactive β-carotene varied greatly. In order to make certain comparisons these data were reduced to the basis of C.P.M./mg./0.1 mc. values. These values were not adjusted to a culture basis. This procedure should be valid unless the radiation from C^{14} affected the fungus. These data are given in Table 13.

The variation in the C.P.M./mg./0.1 mc. values (Table 13) among the experiments was more than threefold. A correlation was sought among the available data on the behavior of the fungus during the 28-hour period during which radioactive β-carotene was synthesized. These data included the amount of mycelium produced, the amount of carotene synthesized, and the amount of glucose utilized (Table 14). These data suggest that the amount of glucose utilized during the 28-hour period was of considerable importance in determining the amount of C^{14} incorporated into β-carotene. In experiments where less than 15 mg. of glucose was used during the 28-hour period when radioactive carotene was being synthesized the C.P.M./mg./0.1 mc. values were high. On the other hand, the C.P.M./mg./0.1 mc. values were low when more than 15 mg. of glucose was utilized during the synthesis of radioactive β-

Table 13. Specific Activity of Radioactive β-Carotene Produced in Different Experiments and the Calculated Specific Activity per Milligram of β-Carotene per 0.1 mc. of Radioactive Acetate

No.	mc. Added	C.P.M. mg.	C.P.M./mg./0.1 mc.
1	2.0	985,000	49,250
2	0.1	39,000	39,000
3	2.0	485,000	24,250
4	0.12	81,000	67,500
5	0.2	132,000	66,000
6	3.0	626,000	20,200

Table 14. The Changes in Weight, Carotene, and Glucose Utilized During the 28-Hour Period During Which Radioactive β-Carotene Was Being Synthesized

No.	Mycelium mg.	Carotene μg.	Glucose Utilized mg.	C.P.M./mg./0.1 mc.
00[1]	1.5	19	8	42,700
0[1]	10.2	76	18	28,800
3	5.0	86	40	24,250
4	4.8	65	12	67,500
5	9.0	18	11	66,000

[1]Data from non-consecutive experiments not otherwise referred to in the text.

carotene. This suggests that the addition of labeled acetate should be delayed until the rate of glucose utilization falls to 15 mg. or less per day.

Acetate appears to enter all of the metabolic pathways of Phycomyces blakesleeanus. Much more remains to be done to define the conditions that channel maximum amounts of acetate into β-carotene. In these experiments from 0.68 *to* 1.93 *per cent of the* C^{14} *utilized as labeled acetate was found in the total quantity of β-carotene synthesized.*

Discussion

The ideal C^{14}-labeled carbon source for the biosynthesis of radioactive β-carotene by *Phycomyces blakesleeanus* (or any other organism) would be readily available, cheap, and completely incorporated into the β-carotene molecule. Such compounds are not known. None of the C^{14}-labeled substrates that have ben used for the biosynthesis of radioactive β-carotene are used for this purpose alone. Sources of carbon such as glucose, fructose, and acetate suffice for the complete carbon nutrition of *P. blakesleeanus*. As a result, when these compounds (uniformly labeled) are used, the C^{14} will be incorporated into all the compounds synthesized, and a considerable part lost to the atmosphere as carbon dioxide. The per cent of C^{14} incorporated into the various compounds and lost as

carbon dioxide will depend on the relative amounts of these products formed. The use of C^{14}-labeled precursors such as mevalonic acid might be expected to reduce the amount of C^{14} incorporated into compounds other than β-carotene. However, mevalonic acid has been reported to be a precursor of such compounds as squalene and sterols. If this is true of *P. blakesleeanus* also, less than 100 per cent of the added mevalonic acid would be incorporated into β-carotene. The existence of multiple pathways of utilization and end-uses of any metabolite, so far found, results in a low percentage of incorporation into β-carotene.

A similar situation obtains when chlorophyll-containing plants are grown in a closed system with radioactive carbon dioxide. Carbon dioxide serves them as the sole source of carbon and as a result C^{14} is incorporated into all of the compounds synthesized. Since the amount of β-carotene synthesized by any organism is but a fraction of 1 per cent of all of the compounds synthesized, the expected yields of radioactive β-carotene synthesized will be low (based on the amount of radioactive carbon dioxide used).

The above discussion emphasizes the limitations in the biosynthesis of β-carotene. While the unavoidable "loss" of C^{14} will be high under any condition there are ways by which this loss may be minimized. The phases of growth and carotenogenesis overlap somewhat in *P. blakesleeanus,* but by the judicious use of the information presented in Part I of this Bulletin and in the publications of others, these may be considered as essentially separate biochemical processes. Under the conditions used in this work, most of the mycelium is produced *before* much carotene is synthesized. This suggests that C^{14}-labeled substrates should be added *after* the production of mycelium is essentially complete. When labeled acetate was added at the time of inoculation this compound was almost completely utilized before the synthesis of much carotene took place. These conclusions are particularly applicable to C^{14}-labeled carbon sources which are used rapidly and completely.

The addition of a C^{14}-labeled substrate that is used completely only by the end of the period of incubation will result in a low level of incorporation of C^{14} into β-carotene. For the purpose of illustration, it will be assumed that uniformly-labeled glucose-C^{14} was added at the time of inoculation of the basal medium, that the carrier and labeled glucose was completely utilized by the time the mycelium was harvested, and the mycelium contained 200 μg. of β-carotene. The 250 mg. of glucose (40% C) in 25 ml. of medium contained 100 mg., or 100,000 μg. of carbon. The 200 μg. of β-carotene (89.5% C) contained 179 μg. of carbon. The per cent of glucose-carbon converted into β-carotene

would be 0.179 per cent, or about one atom of C^{14} out of 560 added at the time of inoculation.

In the second example, it will be assumed that uniformly-labeled glucose is added to the cultures at the time when half of the glucose originally present in the basal medium has been utilized. Only a trace of carotene will have been synthesized by this time (3 - 4 days). It would be expected that twice as much C^{14} would be incorporated into β-carotene under these conditions as when the labeled glucose was added at the time of inoculation.

A number of considerations enter into the choice of a C^{14}-labeled substrate for the biosynthesis of radioactive β-carotene. Among these may be mentioned cost and the efficiency with which C^{14} is incorporated into β-carotene. The C^{14}-labeled compounds, acetate and glucose, were considered. The cost of acetate-1-C^{14}, acetate-2-C^{14} and glucose-u-C^{14} at the time this work was done was $110, $250, and $550 per mc., respectively. If the efficiency with which C^{14} from these three compounds is incorporated into β-carotene is equal, acetate-1-C^{14} would be the cheapest source of C^{14}. In addition, acetate has a decisive advantage over glucose from the standpoint of rapidity of utilization. Under the conditions used in this work, the acetate added during the phase of carotenogenesis was utilized within 24 hours after it was added to the cultures. The utilization of glucose would require many days. In most of the experiments which were terminated after 8 or 9 days some residual glucose remained in the culture medium. The choice between discarding the residual radioactive glucose and recovering it would have to be made.

The justification for adding carrier acetate before and at the time radioactive acetate was added was based on the observation that this procedure resulted in decreased glucose utilization. It was thought that under these conditions acetate would be preferentially utilized for the biosynthesis of β-carotene. The data in Table 14 suggests that more C^{14}-labeled acetate is incorporated into β-carotene when glucose-utilization is low. Lilly *et al.* (1958) showed that the amount of glucose-carbon spared under these conditions was about equal to the amount of acetate carbon utilized. This procedure of adding carrier acetate appeared to reduce the flow of glucose-carbon into β-carotene. A different expedient which had the same aim was used by Grob (1957) who added malonic acid to glucose-grown cultures of *Mucor hiemalis* to inhibit the utilization of glucose before adding mevalonic acid-2-C^{14} for the biosynthesis of radioactive β-carotene.

The suitability of other species of fungi for the biosynthesis of radioactive carotenoid pigments remains to be investigated in detail.

The procedures used in this work with *P. blakesleeanus* would probably require extensive modification before being used with other species. The optimum conditions for the biosynthesis of carotene are known to be different for different species, e.g., *Choanephora cucurbitarum*.

It is unfortunate that none of the published papers on the biosynthesis of radioactive β-carotene appear to contain sufficient information to permit a valid detailed comparison with the results presented in this Bulletin.

Summary, Part II

A detailed procedure has been presented for the production of radioactive β-carotene by an isolate of *Phycomyces blakesleeanus*. While the method is not particularly difficult, it is essential that certain precautions be observed. These include maintaining the cultures from the time of inoculation to harvest at 25° to 26° C. and exposing the cultures continuously to 50 to 60 foot-candles of illumination. Undue agitation of the cultures must be avoided. The equipment required is simple and the elapsed time required to prepare a pure sample of radioactive β-carotene is about two weeks.

The yields of crystalline β-carotene and the specified activity (C.P.M./mg.) have varied among experiments. No failures have been encountered. The specific activity of the radioactive β-carotene may be varied within wide limits. With experience and attention to detail, more than 1 per cent of the radioactivity of the C^{14}-labeled acetate utilized is incorporated into β-carotene.

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